Intersection of Mechanobiology and Musculoskeletal Regenerative Rehabilitation

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Abstract

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Mechanical stimuli play an essential role in the formation of structurally and functionally appropriate cellular and tissue modeling/remodeling. Musculoskeletal system responds to mechanical stimuli through physical movement in an integrated fashion, transferring forces from tissue to cellular and molecular scales. In the context of rehabilitation, mechanical stimuli are referred to as mechanotherapy and physical therapists use them to treat musculoskeletal disease and/or injury, with the goal to modulate cellular behavior and subsequent tissue adaptation. However, the link between mechanotherapy and subsequent cellular and tissue mechanoadaptation is not well established in the literature. To address this, first, recent literature on how mechanotherapies such as exercise training, stretching, electrical stimulation, and shockwave might activate mechanotransductive and metabolic signaling pathways were evaluated. Although

there is sufficient evidence on regulation of these signaling pathways; however, there is insufficient data on the mechanism of activation of these pathways following mechanotherapy in aged, diseased and injured skeletal muscle tissues. In the second research study, mechanical stimulation in the form of passive stretch was used to develop *in vitro* skeletal muscle injury model. Then, the model was applied to both normal and Duchenne Muscular Dystrophy (DMD) skeletal muscle fibers to induce passive stretch-induced injury, and the acute biochemical responses of cytoplasmic and myofibrillar proteins to the selected stretch protocol were measured. The preliminary findings indicated that muscle fibers in DMD were more susceptible to the passive stretch-induced injury protocol than normal. Lastly, in the clinical regenerative rehabilitation pilot study, feasibility and short-term efficacy of rehabilitation program after regenerative therapies was assessed to harness the mechanosensitivity of chondrocyte and better facilitate tissue healing and regeneration. The initial findings demonstrated that the rehabilitation program was clinically meaningful across most Knee injury and Osteoarthritis Outcome Score (KOOS) subscales between 2- and 3-months post injection in both treatment groups. Future randomized clinical trials are needed to fully understand the role of mechanical stimulation of chondrocyte to facilitate the repair and regeneration following regenerative therapies in knee degenerative joint disorders.

Plain Language Summary

The musculoskeletal system responds to mechanical stimulations through physical movement. Physical therapists use interventions that apply mechanical stimulations to treat musculoskeletal disease and/or injury. The link between these interventions and subsequent tissue responses is not well established. To address this, the first paper reviewed recent evidence on how physical therapy interventions might alter skeletal muscle tissue response. There is inadequate evidence on the mechanism of activation of signaling pathways in aged, diseased, and injured skeletal muscle tissues. The second paper describes a basic research study, where mechanical stimulation in the form of stretching was utilized to model skeletal muscle injury. Muscle biochemical response in normal and Duchenne Muscular Dystrophy (DMD) rat skeletal muscle fibers to the selected protocol was examined. The initial findings showed muscle fibers were more prone to injury than normal caused by the passive stretch. The final paper describes a clinical research study, where short-term effectiveness of a rehabilitation program after regenerative therapies was evaluated to better understand the role of physical therapy in acceleration of healing. The preliminary findings demonstrated that the rehabilitation program was clinically meaningful at three months after regenerative therapies when patients' opinions about their knee and associated knee problems such as pain, symptoms, activities of daily living, knee function in sport and recreation, and knee-related quality of life were assessed.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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This dissertation is dedicated to my parents, grandmother and partner who encouraged me to pursue my dreams and finish my dissertation.

Chapter 1. INTRODUCTION

The present hybrid dissertation document contains three linked papers outlined as the scoping review manuscript, basic research study report, and clinical research manuscript. The first paper is a scoping review manuscript that presents an overview of a large body of literature pertaining to common mechanotherapies used in physical therapy practice. It mainly reviewed common mechanotherapies used in physical therapy practice and elaborated on how they activate mechanotransductive and metabolic signaling pathways in skeletal muscle to trigger desired therapeutic effects. The second paper includes a research study report that summarized methods used to harvest and isolate lower extremity skeletal muscles in one-month old rats (DMD and wild-type), steps to optimize skeletal muscle cells culture matrix and environment, and application of passive-stretch via Cytostretcher cell stretching device to induce injury. The third chapter is a stand-alone manuscript that introduces orthobiologics as novel non-operative treatment for degenerative knee chondral lesions followed by the orthobiologics rehabilitation program. The purpose of this paper is to evaluate the short-term effects of the orthobiologics rehabilitation program following orthobiologics injection in individuals with chondral or meniscal lesions.

Intersection of Mechanobiology and Physical Therapy Practice in Skeletal Muscle Rehabilitation

Abstract

Mechanotherapies introduce mechanical force to alter desired tissue response. To develop mechanotherapies, it is crucial to study mechanobiology. Most interventions used in physical therapy provide mechanical stimulation at both the cellular and tissue levels. Mechanotransduction is a foundation of mechanotherapy and developing insight into the specific molecular and cellular skeletal muscle responses to mechanotherapy will allow physical therapists to increase understanding of therapeutic dosing and potentially improve clinical outcomes in patients undergoing musculoskeletal rehabilitation. This scoping review presents overview of current evidence on mechanotherapies frequently used by physical therapists, such as exercise training, blood flow restriction training, stretching, electrical stimulation, and shockwave therapy and further describe how the molecular pathways drive skeletal muscle tissue response to these therapies.

Chapter 2: INTRODUCTION

Mechanotherapy is defined as "any intervention that introduces mechanical forces with the goal of altering molecular pathways and inducing a cellular response that enhances tissue growth, modeling, remodeling, or repair" (Loghmani et al., 2016). This definition encompasses physiologic responses at multiple levels (molecules, cells, tissues) as the mechanism for functional adaptations to mechanical forces. This scoping review narrows this examination to skeletal muscle, a mechanosensitive tissue that generates, absorbs, and transmits forces to produce movement. This tissue is capable of adapting to its mechanical environment, and specific responses at each level occur as a function of specific cellular and molecular signaling pathways.

Two primary signaling pathways directly regulate skeletal muscle function: mechanotransductive and metabolic signaling pathways. Understanding the mechanisms that regulate these pathways is critical to targeting interventions that promote desirable functional outcomes in physical therapy practice. The main purpose of this scoping review is to examine existing evidence for the activation and/or inhibition of the primary signaling pathways modulated by physical therapy interventions. The overarching question to be addressed is, can mechanotherapies that alter molecular and cellular responses elicit skeletal muscle tissue growth to the point that functional improvement has a positive impact on patients' quality of life.

Mechanotransductive Pathways in Skeletal Muscle

An understanding of the therapeutic benefit of mechanotherapy on skeletal muscle tissue function necessitates an understanding of how this tissue senses and converts mechanical force into cellular and molecular responses. This complex process of mechanotransduction is mediated by extracellular matrix proteins, transmembrane integrin receptors, and associated signaling

molecules (Burkholder, 2007). In fact, many musculoskeletal and neuromuscular disorders, ranging from osteoporosis and osteoarthritis to muscular dystrophies and sarcopenia, share the common feature that their clinical presentation results from abnormal mechanotransduction. Additionally, altered or reduced mechanoadaptation is associated with increased susceptibility to sport-related injury risk, with individuals showing less adaptation being at greater risk of injury (Ingber, 2003). Downstream of a mechanical stimulus, signaling is initiated and propagated at three levels within skeletal muscle.

1. Mechanotransduction at the cellular level

Mechanotransduction starts with the transmission of force to a cell's microenvironment (mechanocoupling) and the activation of cell sensory machinery known as mechanosensitive receptors (Figure 1A). These mechanosensitive receptors include integrins, stretch-activated ion channels, growth factor receptors, and G-protein-coupled receptors.

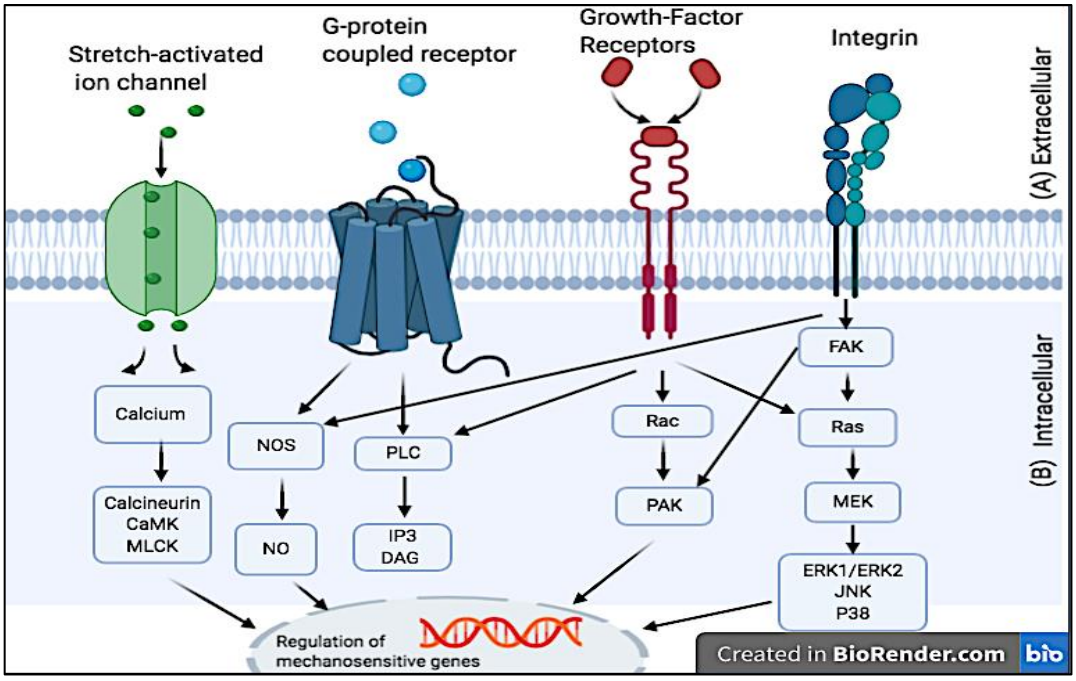

Figure 2.1: Mechanotransduction at the cellular (A) and the molecular (B) levels.
CaMK=calcium/calmodulin-dependent kinase; DAG=diacyl-glycerol; ERK= extracellular signal-regulated kinase; FAK=focal adhesion kinase; IP3=inositol triphosphate; JNK=c-Jun N-terminal Kinases; MEK=mitogen-activated protein kinase; MLCK=myosin light-chain kinase; NO=nitric oxide, NOS=nitric oxide synthase; PAK= p21-activated kinase; PLC= phospholipase C; Ras=rat sarcoma small GTPase.

Integrins are transmembrane receptors that physically connect the intracellular space to the extra cellular matrix (ECM) proteins through transmembrane associations with actin cytoskeletal structures allowing for force transmission and intracellular biochemical signal regulation. Focal adhesion complexes (FAK) forms the bridge between cytoskeleton filaments and the nucleus. In the integrin's resting state, during periods of minimal muscle activity, the extracellular head sits in a bent conformation blocking its interaction with extracellular ligands. Upon a stimulus such as exercise, the extracellular head swings straight, the two integrin subunits separate, and extracellular protein binding and cellular force propagation follow. Integrin is highly sensitive to mechanical perturbation; however, maximal muscle activity such as high intensity exercise training is not required for integrin recruitment (Schwartz, 2010).

2. Mechanotransduction at the molecular level

Following the physical transduction of force, the conversion of mechanical signal into a biochemical response (biochemical coupling) involves the recruitment of mechanosensitive molecular signaling pathways. These pathways are composed of an overlapping cascade of cytosolic mediators that transmit the biochemical signal from the cell surface to the nucleus (Figure 1B). Following stimulation of integrin, Mitogen-activated protein kinases (MAPKs) pathway including, JNK, ERK1/2, and p38 proteins, become activated and act as the point of convergence for a variety of signaling cascades regulating skeletal muscle mechanotransduction gene expression. Additionally, stretch-activated ion channels initiate downstream molecular pathways including, Calcium/calmodulin-dependent kinase (CaMK), Calcineurin, and Myosin light-chain kinase (MLCK), which are sensitive to intracellular Ca^{+2} ion concentration. Ca^{+2} signaling in skeletal muscle triggers diverse cellular responses required for myogenesis and skeletal muscle

homeostasis. Stretch-activated ion channels may be particularly important when the exercise involves eccentric contractions. In fact, stretch-activated channels could contribute to modulating stretch or eccentric exercise-induced cell growth in skeletal muscle (McBride et al., 2000).

3. Mechanotransduction at the tissue level

The final phase of mechanotransduction is the effector response. Once the biochemical signal is transmitted to the nucleus, it further induces expression of mechanosensitive genes. Moreover, the biochemical signal may propagate intercellularly to affect adjacent cells or pass into the extracellular space to influence synthesis or degradation of the ECM. The ultimate effector functions are reflected in cell-cell and cell-matrix connections, cell proliferation and differentiation, ECM remodeling, and cell migration. Mechanotransduction may be altered through changes in cell mechanics, extracellular matrix structure, or even by deregulation of the molecular mechanisms which sense mechanical signals and convert them into a biochemical response in the first place. An understanding of mechanotransduction regulation is important because the molecules that mediate mechanotransduction may represent future target mechanisms for therapeutic intervention in a variety of musculoskeletal diseases (Khan & Scott, 2009).

Besides mechanotransductive pathways, metabolic pathways have significance in mediating skeletal muscle responses to therapeutic interventions aimed at prevention of muscular weakness following decreased mobility and/or immobility caused by musculoskeletal disorders. These metabolic pathways are briefly introduced below and further reviewed in existing evidence on how these metabolic pathways are regulated by different types of mechanotherapy in physical therapy practice.

Metabolic Pathways in Skeletal Muscle

Skeletal muscle responds to increase and decrease in mechanical stimulation through various physiological adaptive mechanisms. The balance between skeletal muscle protein synthesis and degradation is a determining factor for muscle size and function. Three primary signaling pathways control skeletal muscle metabolic functions: Mammalian Target of Rapamycin (mTOR), AMP-Activated Protein Kinase (AMPK), and Peroxisome proliferator-activated receptor-c coactivator-1a (PGC-1α) (Olsen, et al., 2019). The mTOR signaling pathway is identified as the master regulator for anabolic protein synthesis, increasing skeletal muscle size particularly under mechanical stimulation. However, not all mechanical stimuli are capable of activating this pathway, and among those that are, there are differences in the magnitude of activation. This scoping review examines these differences in more details.

AMPK has regulatory effects on fatty acid oxidation and glycogen metabolism in skeletal muscle; it also regulates muscle protein degradation, a process which serves as a source of amino acids that can be used for energy production. This pathway also functions as a sensor for the energy status of a cell, as AMPK becomes activated when the AMP/ATP ratio is high, triggering a wide range of catabolic pathways directed to increase cellular levels of ATP (Garcia & Shaw, 2017). PGC-1a is a critical regulator of mitochondrial biogenesis and function that controls oxidative metabolism. Activated AMPK upregulates catabolism via mitochondrial biogenesis and function, thus PGC-1α activity is necessary for AMPK-mediated mitochondrial activation (Fernandez-Marcos & Auwerx, 2011).

Overall, to better understand how regulation of mechanotransductive and metabolic pathways impact skeletal muscle tissue function in the context of mechanotherapy, the subsequent

sections review the current evidence related to the underlying mechanism of activation of these pathways following exercise training, blood flow restriction training, stretching, electrical stimulation, and shockwave therapy.

Mechanotherapy

1. Resistance Exercise Training. It has been well established that resistance exercise induces an increase in skeletal muscle protein synthesis and hypertrophy (Hornberger, 2011). Skeletal myofiber hypertrophy as an adaptation to resistance exercise training is accomplished via cellular protein accretion over time, which requires increased ribosomal function and consequently protein translation. Both of these processes are highly regulated by the mTOR signaling pathway (Bamman et al., 2018). Increasing evidence shows that the mTOR signaling pathway integrates with MAPKs at several points that lead to hypertrophic response (Hawley et al. 2014) (West et al., 2016). In fact, resistance exercise is a potent stimulator of MAPKs; however, a minimum threshold of intensity is required to activate ERK1/2 and p38, both members of the MAPK family (Burd et al., 2010; Holm et al., 2010). Another study showed that JNK is the most mechanosensitive MAPK, and its activation corresponds to increasing resistance exercise intensity (Gehlert et al., 2015). In general, the activation of MAPKs is highly dependent on resistance exercise parameters. For example, it has been demonstrated that high-intensity, low-repetition resistance exercise programs caused higher activation of ERK1/2 and p38 compared to low-intensity, high-repetition exercise (Hulmi et al., 2012). Despite the plethora of data on short-term MAPK activity in the context of resistance exercise, there is a lack of data on the role of MAPKs in chronic exercise adaptations in humans. Despite their known role in regulating mechanotransductive signals, more research is warranted to fully elucidate the MAPK role in long-term adaptations to resistance exercise in human skeletal muscle.

Several factors play a role in satellite cell activation, contributing to the hypertrophic response to resistance exercise. Each nucleus in the multinucleated myofiber is limited in its capacity to transcriptionally regulate a fixed volume of cytoplasm, the myonuclear domain. Thus, significant myofiber hypertrophy, beyond the domain ceiling of each myonucleus, requires the addition of nuclei. These additional nuclei are believed to result from differentiation and integration of satellite cells into myofibers (Bamman et al., 2018). Previous human studies showed that a significant increase in satellite cells number occurs during the first 24 hours following acute resistance exercise of lower extremity muscles; those counts continued to be elevated at 72-96 hours, then declined afterwards, with a highly intensity-dependent acute response (MacKay et al., 2009; Martin et al., 2012). The acute response was minimal with exercise intensity lower than 40% of the 1 repetition maximum (RM), but was 2-to 3-fold higher with intensity above 60% of 1 RM (Kumar et al., 2009). In similar fashion, chronic resistance exercise training in human studies (with high intensity compared to low intensity protocols) showed a significant increase in satellite cell proliferation in response to training periods between 9 to 16 weeks (Sakamoto et al., 2004; Ishido et al., 2006; Carey et al., 2007; Akiho et al., 2010; Mackey et al., 2011; MacKenzie et al., 2013). Therefore, evidence support satellite cell activation during muscle hypertrophy, where the number of myonuclei in each fiber increases as satellite cells multiply and fuse with the existing muscle fibers in order to support the larger cytoplasmic volume.

In contrast to this myonuclear addition theory, a few investigations reported muscle fiber hypertrophy in the absence of satellite cell-mediated myonuclear accretion (McCarthy et al., 2011; Lee et al., 2012; Fry et al., 2014). However, more recent studies argue that hypertrophic response to mechanical overload is highly dependent on satellite cells (Egner et al., 2016; Goh et al., 2017). In summary, mechanical load is a key hypertrophic stimulus associated with resistance exercise.

Hypertrophy occurs via cellular protein accretion over time, which requires increased protein translation and is highly regulated by the mTOR signaling pathway in conjunction with MAPKs including ERK1/2, p38, and JNK. Subsequent hypertrophy depends on myonuclear addition via satellite cell activation. Although initial myofiber enlargement may occur primarily by protein accretion, satellite-cell mediated myonuclear addition may be essential for continued hypertrophy as the skeletal muscle continues to be exposed to the mechanical stimuli of resistance exercise.

2. Endurance Exercise Training

While resistance exercise induces hypertrophy, endurance exercise primarily affects oxidative capacity in skeletal muscle tissue. Mitochondrial biogenesis, an adaptive response to endurance exercise, leads to an increase in the number of mitochondria which in turn enables greater oxygen utilization to produce ATP (Leick et al., 2010). PGC-1α is recognized as a primary regulator of oxidative metabolism that drives adaptive mitochondrial responses to endurance exercise in muscle tissue. Moreover, AMPK signaling is involved in long-term metabolic changes in mitochondrial biogenesis and thereby promotion of an oxidative muscle phenotype (Martinez-Redondo et al., 2015). p38 MAPK and Ca^{2+}/calmodulin are the two signaling pathways in the process of PGC-1a expression following endurance exercise (Fernandez-Marcos & Auwerx, J., 2011).

Increased numbers of satellite cells have been described after endurance training in both young and old human subjects following acute and chronic endurance exercise training, without significant change in fiber diameter or myonuclear content. (Saleem et al., 2014). Current evidence suggests that the intensity of aerobic exercise, rather than duration, is a fundamental driver of satellite cell expansion. Some studies reported an increase in the satellite cell number following intense aerobic intermittent cycle ergometer training (Kurosaka et al., 2009; Charifi et al., 2003),

while others reported no significant changes when using a low-to-moderate relative exercise intensity (75% of the individual baseline VO_2-max) (Snijders et al., 2011).

In summary, resistance and endurance exercise have long been known as effective stimuli for hypertrophy and increased aerobic capacity, respectively. Both interventions induce these adaptations in part via satellite cell activation. Although it has been established that hypertrophy results from a combination of cellular protein accretion and satellite-cell mediated myonuclear addition, the effects of aerobic exercise on satellite cell function are less understood. Exercise training is accessible, with easily manipulated parameters, which makes this non-pharmacologic stimulus a promising intervention in musculoskeletal rehabilitation. Exercise, as a physiologic stressor, exerts modulatory effects on different stages of the satellite cell life cycle, although further investigation is required to better define the response to different modes of exercise.

3. *Blood Flow Restriction Training*

Blood flow restriction (BFR) is a training method partially restricting arterial inflow and fully restricting venous outflow in working muscle during exercise. Previous evidence has demonstrated that low-load resistance training with 20%–50% of 1RM combined with blood flow restriction can produce significant hypertrophy and strength gains; however, the underlying mechanisms of action are still not completely explained (Takarada et al., 2000; Takarada et al., 2004; Burgomaster et al., 2003; Loenneke et al., 2015). It is suggested that a greater accumulation of metabolites following blood flow restriction-induced hypoxic stimulus, may act as a primary moderator of the anabolic response to this form of exercise (Schoenfeld at al., 2013). Similar to high-load strength training, BFR has been reported to cause long lasting (~24 h) elevations in mTOR pathway activation (Abe et al., 2012) and elevated myofibrillar protein synthesis together

with reduced expression of myostatin, a key negative regulator of muscle mass (Bjørnsen et al., 2019). BFR training has important implications for individuals who cannot tolerate the mechanical stress of heavy-load exercise, such as post-operation rehabilitation patients and the elderly. From a clinical musculoskeletal rehabilitation perspective, training with low loads may provide one strategy to maximize hypertrophy when heavy loads are not feasible. Low-load BFR training compared with low-load training is equally tolerable and more effective; therefore low-load BFR represents a superior clinical rehabilitation tool. There is a need for an individualized approach to training prescription to minimize patient risk and increase effectiveness (Hughes, 2017).

4. Stretching

Physical therapists frequently use techniques, such as passive range of motion, passive stretching by a therapist, splinting, and serial casting to reduce stiffness and pain, and to restore normal mobility. The elongation of shortened tissue is often the goal of initial therapeutic interventions. cellular and molecular mechanism of adaptation of skeletal muscle fiber to stretching may provide the basis for its clinical application. For example, prolonged passive stretches of skeletal muscle such as splinting and/or serial casting have been implicated in inhibiting muscle atrophy following orthopedic interventions. The number of sarcomeres in series is normally regulated to set optimal fiber length- the length at which the maximum number of cross bridges and thereby maximum force output is possible. It has been shown that prolonged muscle elongation can lead to longer muscle fibers with more sarcomeres in series (myofibrillogenesis). The underlying mechanisms of myofibrillogenesis may be as follows: 1) the phosphorylation of integral membrane proteins and associated cytoskeletal molecules through activation of mechanotransductive pathways such as FAK and MAPKs, 2) the secretion of selective growth factors, such as IGF-1 and HGF, 3) changes in the intracellular calcium through activation of

stretch-activated calcium channels and subsequent formation of CaKM, nitric oxide synthase (NOS) activity, and nitric oxide (NO) production (De Deyne, 2001).

There are factors to consider when clinicians evaluate the efficacy of passive stretch on muscle length regulation, because not all passive stretch can trigger opening of stretch-activated calcium channels. One factor is the position of optimal length of target muscle. The existing optimal length of target muscle might be maladapted, by muscle contracture or limited joint range of motion, to a shorter length; thus, in this case passive stretch beyond the existing optimal length might be sufficient to activate stretch-activated calcium channels conducive to myofibrillogenesis. However, increased pain during passive stretch may further limit the percentage of lengthening. Therefore, the effects of passive stretching vary within subjects (Riley & Van Dyke, 2012).

Additionally, passive stretching is involved in the activation of satellite cells via regulation of MAPKs signaling (15). A cascade of biochemical events, including CaKM formation (Tatsumi et al., 2009), activation of mechano-sensitive ion channels (Formigli et al., 2007), nitric oxide synthase (NOS) activity, nitric oxide (NO) production, IGF and HGF secretion (Morrissey et al., 2016) are involved with stretch-mediated activation of satellite cells. The addition of more sarcomeres could be the result of the proliferating satellite cells fusing with preexisting muscle fiber cells. Nunes et al. performed an extensive literature search to evaluate whether stretch training is feasible to induce muscle hypertrophy in humans. The available literature indicates that passive, low-intensity stretch does not appear to trigger beneficial changes in muscle size (Nunes et al., 2020). Alternatively, current evidence suggests that intense stretch training, particularly when combined with active muscle contractions, may elicit muscle hypertrophy, though the relative paucity of research implementing such protocols precludes the ability to draw clear conclusions. Future studies using high-intensity passive stretch training protocols should be carried out to fill

existing gaps in research and provide better insight into the potential adaptations and their practical applicability.

5. Electrical Stimulation

Neuromuscular electrical stimulation (NMES) has long been considered as a "peripheral" therapeutic modality for maintaining or rebuilding skeletal muscles during/after a period of reduced use and can be applied as a complementary intervention to voluntary exercise training. NMES is a rehabilitation modality that can be used to mimic the physiologic action of neurons and recreates the mechanical environment experienced by resident muscle cells through induced contraction of innervated muscle fibers. The transmission of electrical signals via nerve innervation is a well-known contributor to directing the differentiation of skeletal muscle cells by activating phosphoinositide 3-kinase (PI3K), G-proteins, and AMPK (Handschin et al., 2015). *In vitro*, tissue-engineered skeletal muscle constructs were stimulated electrically with continuous electrical pulses to artificially mimic exercise-training response and results showed increased cellular proliferation and differentiation (Ito et al., 2014). Guo et al studied the effect of *in vivo* electrical stimulation on disuse muscle atrophy in mice and concluded that the loss of myonuclei and satellite cells could be rescued by maintaining a satellite cell pool for subsequent muscle regeneration (Guo et al., 2012).

Therefore, NMES can be used to stimulate increased cellular proliferation, desired differentiation, and improved skeletal muscle function (Guo et al., 2012; Distefano et al., 2013; (Filippo et al., 2017). Moreover, NMES may act as an efficient protector of muscle competence when subjects are unable to engage in resistance or aerobic training programs. Additionally, this method is usually employed as a passive "substitute" of dynamic training and an "exercise emulator," since NMES activates PGC-1α as well as mTOR pathways (Veldman et al., 2016).

Therefore, this strategy could be particularly useful in patients with paraplegia, tetraplegia, obesity, limited mobility, frail elderly, or any person needing prolonged bed rest.

6. Shock Wave Therapy

Low-energy Extracorporeal Shock Waves Therapy (ESWT) exerts a mechanical force on cell membranes and contents, thereby activating mechanotransduction. One cellular membranous signaling pathways highly modulated by ESWT is the FAK pathway. Low-energy shock waves interact with integrin and activate FAK by phosphorylation mediated by integrin α5 and β1, thereby triggering a series of cellular signaling and related biological changes. mTORC1 pathway is regulated by FAK phosphorylation in the context of Li-ESWT mediated mechanotransduction. Li- ESWT induces mTORC1 phosphorylation and subcellular translocation, which in turn leads to mTORC1 mediated control of cell proliferation. (Liu et al., 2019).

In the first *in vitro* study, human skeletal muscle cells were exposed to radial extracorporeal shock, isolated muscle cells were Pax 7 (a marker of satellite cells) as well as for the muscle cell markers NCAM, MyoD, and Myf5. (Mattyasovszky et al., 2018). In an *in vivo* animal model of muscle injury, ESWT demonstrated a significant upregulation of muscle markers MyoD and Myosin, suggesting positive effect on muscle regeneration by regulating satellite cells. ESWT also enhances the blood flow in muscle immediately after application, thus repetitive ESWT use might prolong the healing window and therefore be well suited to treat acute muscle injury or chronic muscle disorders (Langendorf et al., 2020).

Summary

The scoping review pursues to present an overview of available literature on mechanotherapy used by physical therapist, from tissue to cell scale mechanotransduction. Currently, it is still not completely possible to apply mechanobiology for diagnosis and treatment of biological systems, nevertheless, we can apply some mechanical stimulations in the form of mechanotherapies in musculoskeletal rehabilitation. Two primary signaling pathways are described as key underlying regulatory mechanism of skeletal muscle fiber function: mechanotransductive and metabolic signaling pathways. Mechanotherapy employs activation of one or a combination of these signaling pathways in any given therapy to mediate its effects. In clinical practice, skeletal muscle adaptation is distinct for an endurance versus resistance exercise training. Muscle adaptation to endurance exercise training is mediated mainly via effects on satellite cell oxidative capacity and mitochondrial biogenesis and are most robust for moderate-high intensity exercise parameters compared to lower intensity exercise parameters. Muscle adaptation to resistance exercise is mediated mainly via altered protein synthesis and, in contrast to endurance/aerobic training, promotes marked increase in satellite cell number and muscle mass. While observations such as these help to inform exercise prescription, there is insufficient data on the mechanism of activation of these signaling pathways following exercise training in aged, diseased and injured skeletal muscle tissue. Mechanisms other than satellite cells activation might play role in preventing a decrease of skeletal muscle mass in these conditions. Future research should be directed towards exploring the precise mechanisms behind exercise training-induced activation of satellite cells in aged, diseased and injured skeletal muscle tissue in order to better inform clinical practice.

With an enhanced awareness of how mechanobiology directly translates to clinical application, perhaps we can add to our understanding of therapeutic dosing while contributing to improved clinical outcomes of novel therapeutic approaches. As the field of mechanotherapy develops, and new therapeutic approaches being introduced into clinical practice, questions related to specificity, selectivity, and timeline of application of mechanical forces that could trigger optimal cellular response conducive to therapeutic effects remains to be answered. Although, the link between movement biomechanics, physical therapy, and subsequent cellular and tissue mechanoadaptation is not well established in the literature, understanding of these adaptive cellular responses to physical forces will lead to therapeutic reasoning and modification of practice according to the biologic response to interventions. Individualized treatment plans should be developed by therapist and adjusted as frequently as required. In fact, the patients' progress through rehabilitation should be based on assessment of tissue response, strength, and functional abilities/limitations. The correct dosage of mechanotherapy applied during the correct phase of healing may have a profound impact on therapeutic outcomes.

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A Passive-Stretch Induced Skeletal Muscle Injury Platform for Disease Modeling and Drug Discovery

Abstract

Purpose: Inactivity or lack of mobility following chronic skeletal muscle dysfunction in DMD usually causes compromised soft tissue and decreased joint range of motion. Passive stretch techniques in combination with an exercise program are used as therapeutic interventions to prevent musculoskeletal complications in children with DMD. However, the exact role of stretch-based rehabilitation methods is not well established in children with DMD. *mdx* mouse models demonstrated exacerbation of DMD pathology following stretch and exercise training especially eccentric exercise which elicits lengthening contractions. Previous studies mainly examined the effects of eccentric contractions on the mechanical events associated with initiation of skeletal muscle injury *in situ* or *in vivo.* In fact, the underlying molecular and cellular mechanisms of how stretch-based rehabilitation methods in dystrophin-deficient muscle fibers might worsen the disease phenotype have not been fully explained. Therefore, the primary purpose of this study was to establish an early *in vitro* stretch-induced injury model in normal and dystrophic rat skeletal muscle fibers.

Method: Normal and dystrophic primary rat myoblasts were platted on two (control and experimental) nanopatterned Cytostretcher^{TM} chambers. In parallel experiment, dystrophic primary rat myoblasts were transduced with microdystrophin (MOI= 10,000 vg/cell). Myoblasts were allowed to differentiate into multinucleated myotubes for a 4-day period during which media was changed every other day. Myofibers were subjected to passive stretch on day three of differentiation. The experimental setting was replicated three times and samples of differentiation media (N=8) were collected. A commercial electrochemiluminescence assay Panel 1 rat kit (Meso

Scale Discovery, Gaithersburg, MD, USA) was used to quantify concentrations of skeletal muscle-specific injury biomarkers, FABP3, Myl3, and STnI, at two time points in the four groups (WT-stretched, DMD-stretched, WT-control, DMD-control). Two-way analysis of variance (ANOVA) with repeated measure test in GraphPad Prism 9 was used to compare calculated mean of the three biomarkers between two time points.

Results: STnI concentration was significantly higher on day four post differentiation compared to day three in the DMD-stretched group (P=0.0188); whereas STnI concentration in all three groups did not show significant changes between the two time points. FABP3 concentrations showed significant increase on day four of differentiation compared to pre-stretch (day three) in both the DMD-stretched (P= 0.0001) and the WT-stretched (P= 0.0072) groups. However, MyL3 concentration decreased across all groups on day four of differentiation compared to day three. In DMD-stretched and DMD-control groups that were transduced with microdystrophin, STnI concentration was significantly increased between two time points in DMD-stretched group (P=0.0188), while DMD-control group did not show significant change of concentration. However, FABP3 concentrations were not significantly different in both the DMD-control and the DMD-stretched groups.

Conclusion: The initial findings of this study supported the hypothesis that the passive-stretch protocol used caused greater magnitude of injury in dystrophic myofibers compared to normal. However, the preliminary findings did not support the hypothesis that dystrophin deficiency could worsen the response to passive-stretch induced injury. These findings will guide development of future *in vitro* studies for disease modeling, drug discovery and to better understand molecular mechanism(s) responsible for adaptation to exercise-induced muscle injury.

Chapter 3. INTRODUCTION

Skeletal muscle tissue continuously adapts to changes in its mechanical environment through modifications in mechanotransductive gene expressions. However, mechanical stresses that exceed muscle capacity to adaptation usually lead to acute muscle injury. Skeletal muscle injury is characterized by immediate weakness and slowly developing stiffness which is common following eccentric contractions (Tidball, 2011). Mechanical and biochemical markers have been used to explain mechanism of muscle injury and measuring changes in each marker have shed light on alteration that occur in muscle following injurious event. Early mechanical changes include disruption of sarcomeres, disruption of cytoskeletal elements involved in force transmission such as desmin and dystrophin, damage to the muscle sarcolemma, impaired excitation-contraction coupling, and loss of force production. Following the initial disruption of muscle cells, resting intracellular calcium levels increase, inflammatory cells accumulate, and myofibrillar and other proteins become degraded. The histological changes of muscle damage are gradually repaired over several weeks and can involve the regeneration of damaged skeletal muscle fibers from satellite cells

Dysregulation of mechanotransductive pathways involved in the regulation of stretch-activated transmembrane channel and integrin impede skeletal muscle adaptive response to mechanical stress imposed on the membrane. This has significance in the pathophysiology of neuromuscular disorders such as Duchenne muscular dystrophy (DMD) (Yeung & Allen, 2004). While the primary cause of DMD is the absence of dystrophin, pathways which link the absence of dystrophin to skeletal muscle damage are unclear. Dystrophin is a cytoskeletal protein which links intracellular cytoskeleton to a group of proteins in the cell membrane, the dystrophin-associated protein complex, contributing to membrane strength so that in its absence mechanically

induced defects are worse. The absence of dystrophin leads to absence of the dystrophin-associated protein complex, instability of muscle cell membrane and uncontrolled influx of calcium and efflux of muscle-derived proteins. This pathophysiology initiates a set of downstream pathological processes that ultimately lead to loss of muscle cell proteins, myofiber damage and progressive muscle wasting (Mercuri et al., 2013). It is established that elevated intracellular calcium contributes to membrane weakness and increased membrane permeability by either damage to phospholipids caused by activity of phospholipase A_2 or by excessive production of Reactive oxygen species (ROS) leading to lipid peroxidation. Thus, increased membrane permeability is identified as a secondary consequence of elevated intracellular calcium and reactive oxygen species in DMD pathology (Allen, Zhang & Whitehead, 2010).

The degenerative process impedes the capacity of muscle fibers to regenerate, and they are gradually replaced by fibrotic and adipose tissues. As the number of muscle fibers decreases, muscle contractility declines, which contributes to the development of muscle weakness. Progressive weakness leads to loss of independent ambulation, respiratory insufficiency, and reduced life expectancy, with death typically occurring in the third or fourth decade of life due to cardiorespiratory complications (Kieny et al., 2013).

In this patient population, effective therapies aim at improving skeletal muscle function remain largely unknown. The current standard of care in DMD is an interdisciplinary approach combining rehabilitation and drug therapies, although there is no definitive cure for DMD. Corticosteroid therapy has been used in treatment of DMD, which only delays the downward progression of the disease and does not restore the normal physiologic function of the dystrophin-deficient muscle fibers (Angelini, 2007). Despite beneficial effects of prolonged ambulation and improved cardiopulmonary function, corticosteroids are often discontinued due to various side

effects ranging from weight gain to pathologic bone fractures (Connolly et al., 2002). From the rehabilitation perspective, the safety and efficacy of exercise training in children with DMD have been long debated. In individuals with DMD, there has been a considerable controversy regarding the use of exercise, especially lengthening contraction (Gianola et al., 2013; Kostek & Gordon, 2018; Spaulding & Selsby, 2018).

Evidence from *mdx* models has demonstrated exacerbation of muscle pathology following exercise training. Some mouse studies have shown that dystrophic muscles have an increased vulnerability to high mechanical forces such as eccentric exercise (Okano et al., 2005; Carter et al., 2002). Dystrophic muscles have distinctive structural vulnerabilities which predispose them to damage from eccentric exercise. The lack of dystrophin in DMD prevents stabilization of the sarcolemma during muscle contractions and the proper transmission of mechanical force (Kobayashi at al., 2008; Markert at al., 2011). Studies of dystrophic *mdx* muscle fibers have determined that without membrane stabilization by dystrophin, there is sarcolemma tearing, dysfunction of stretch-activated channels, and mis localization of calcium channels leading to calcium influx (Fraysse et al., 2004; Yeung et al., 2005; Constantin et al., 2006).

Eccentric contraction causes a rise in the intracellular calcium by activation of stretch-activated channels. This increase in intracellular calcium leads to loss of membrane strength and/or damage to the lipids which are the most possible underlying causes of increased membrane permeability (Allen, Zhang & Whitehead, 2010). Chronic high intracellular calcium levels stimulate mitochondrial production of reactive oxygen species, overwhelming the antioxidative response and causing further damage. Alternatively, calcium activates calpain-mediated proteolytic activity to induce cell death in damaged dystrophic myofibers (Shkryl et al., 2009). Taken all together, *mdx* mouse exercise studies concluded that eccentric exercise was

contraindicated in patients with DMD. In fact, a lack of *in vivo* studies examining effects of mechanical loading on skeletal muscle fibers contributes to unclear understanding of the most effective and safe exercise parameters for children with DMD.

Both small and large animal species deficient for dystrophin have been described and have been extremely useful for pre-clinical studies of DMD. The most commonly used small DMD animal model is the *mdx* mouse. The genetic defect in the *mdx* mouse is identical to that of patients with DMD, but there are substantial phenotypic differences between the *mdx* mouse and human patients. For example, in humans, DMD is characterized by progressive decline in muscle function leading to early death. In contrast, the *mdx* mouse experiences an acute muscle necrosis at 3-5 weeks of age, followed by stable muscle dysfunction until approximately 18 months of age at which time progressive histopathologic changes start (Blat et al., 2015). The phenotype of *mdx* mouse is generally milder than the human condition and the underlying causes for the relatively mild *mdx* phenotype are not well understood. The relatively mild *mdx* phenotype has raised concerns about the degree to which findings in this model will translate to humans, thereby making the prediction of drug safety and efficacy in humans challenging (Partridge, 2013).

On the other hand, the golden retriever muscular dystrophy (GRMD) dog is a better model in studies of DMD pathogenesis and treatment development because the GRMD clinical syndrome is more severe than in mice and better resembles the progressive course of DMD, thus canine studies may translate better to humans. While the GRMD model is more clinically relevant than the *mdx* mouse, usage has been limited by practical considerations related to individual variability, high maintenance cost and the number of available dogs (Kornegay, 2017). A novel small animal model, DMD^{mdx} rat, has recently been developed and well characterized with phenotypic properties very close to the human DMD pathology. This model could represent a valuable

alternative model to mice for two main reasons. First, rats are 10 times bigger than mouse and could better reflect the pathological and functional abnormalities observed in DMD patients. Second, rats are still small enough to allow design of studies with high statistical power (Larcher et al., 2014). Therefore, use of new animal model such as DMD ^{mdx} rat could represent a valuable model for pre-clinical study of drug development in DMD. For this reason, we chose to use DMD mdx rats instead of mice to better represent clinical symptoms of patients with DMD.

Previous studies mainly examined the effects of eccentric contractions on the mechanical events associated with initiation of skeletal muscle injury *in situ* or *in vivo* (Warren et al., 1993; Brooks et al., 1995; Brooks, 1998; Gosselin& Burton 2002). No studies have examined the direct effects of passive stretch-induced injury on acute biochemical response of dystrophic rat myofibers *in vitro*. Taken all together, the underlying molecular and cellular mechanisms of how mechanical loading of dystrophin-deficient muscle fibers might worsen the disease phenotype have not been fully explained. Therefore, the purpose of this study is to establish an early *in vitro* passive-stretch induced injury platform in dystrophic and normal rat skeletal muscle fibers. It is hypothesized that dystrophin-deficient rat skeletal muscle fibers are more susceptible to acute injury after passive stretch compared to normal. Furthermore, it is hypothesized that transduction of dystrophin into DMD^{mdx} rat skeletal muscle fiber causes a reduction in the magnitude of stretch-induced damage, showing that the absence of dystrophin exacerbates muscle damage.

Chapter 3. METHODS

The following section explains the methods used for this research study. Approval of rodent animal use was obtained from the University of Washington Institutional Animal Care and Use Committee (IACUC # 443302) to conduct the study. To harvest the myogenic cell population for *in vitro* skeletal muscle growth, differentiation, and mechanical stimulation experiments, three different myoblast isolation protocols were examined. Details of each protocol and findings are explained below.

3.1 Lower Extremity Skeletal Muscle Harvest

3.1.1 Myoblast Isolation Protocol (1)

The first protocol used for lower extremity skeletal muscle harvest and myoblast isolation was adopted from Dr. Maura Parker, research scientist at the Fred Hutchinson Cancer Research Center. Two 9-months old Sprague-Dawley, WT and DMD ^{mdx} rats, were dissected for skeletal muscle harvest and myoblast isolation. The details of the protocol are as follows:

1. The harvested muscle tissues were removed from rat hindlimbs and placed in a 10-cm plate. Connective tissue and fat were removed as much as possible.
2. Muscle pieces were removed, and dab dried on sterilized paper towel or Kim Wipe. Pieces were transferred to a new 10-cm plate. Using 2 scalpels, muscle tissues were chopped using opposing strokes, and continued until the muscle adopted a thick paste-like appearance.
3. Muscle tissues were transferred to 15-ml conical polystyrene tubes containing 400 units/ml of Collagenase Type 4 (Worthington Biochemical; catalog # LS004186) in Dulbecco's modified eagle's medium (DMEM) high glucose.

4. Tubes were shaken vigorously, and incubated tubes were kept in a 37° water bath for 60 minutes.
5. Using a 5-ml plastic Pasteur pipette, muscle was triturated by pipetting up and down 20 times.
6. Contents of the two 15-mlconical tubes were transferred to a single 50-ml conical polystyrene conical tube. The sample was triturated by passing the sample 20 times through a 16½ gauge needle attached to a 20-ml syringe and the same procedure was repeated using an 18½ gauge needle attached to a 20-ml syringe.
7. A 70-µm cell strainer was placed in a 50-ml conical polystyrene tube and the membrane was wetted with 5 ml of D-PBS. 10 ml D-PBS was added to the muscle slurry and applied the mixture to the membrane using a 5-ml pipette or a plastic Pasteur pipette.
8. A 40-µm cell strainer was placed in a 50-ml conical polystyrene tube and wetted the membrane with 5 ml of D-PBS. After sample was passed through strainer completely, the membrane was washed with at least 5 ml of D-PBS.
9. The filter was removed, and enough D-PBS was added to bring the total volume to 50 ml. The sample tube was centrifuged at 1500 rpm for 10 minutes at room temperature.
10. The supernatant was carefully removed by aspiration and the tube was flicked vigorously to dislodge the cell pellet. The cell pellet in 10ml of D-PBS was resuspended and transferred to a 15-ml conical polystyrene tube.
11. The tube was centrifuged at 1000 rpm for 5 minutes at room temperature. The supernatant was removed by aspiration and the tube was flicked to dislodge the cell pellet.
12. The isolated cells were counted using a hemocytometer.

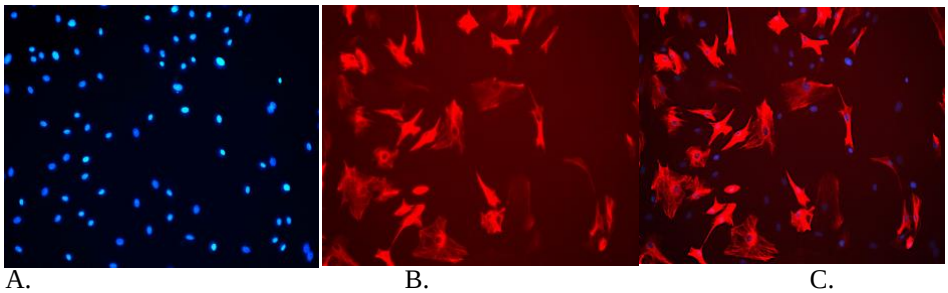
The protocol described above consisted of multiple mechanical dissociation steps along with an enzymatic digestion using Collagenase IV. This protocol required passing the muscle slurry through 40 μm and 70 μm cell strainer, therefore eliminating myogenic progenitors that are attached to the myofibers. Although myogenic progenitors can be isolated directly from the mononuclear cell population, previous studies resulted in a low yield of 1-2x10^5cells from hindlimb muscles of one mouse through direct isolation of myogenic progenitors from skeletal muscle slurry (Yi & Rossi, 2011; Motohashi et al., 2014). It was observed that the harvested tissues were too digested, thus no yield was achieved using this protocol, therefore it was abandoned, and another isolation protocol was trialed.

3.1.2. Myoblast Isolation Protocol (2)

Previous studies showed that satellite cells within the basal lamina of myofibers preserve their quiescent state in low serum media, while they proliferate and outgrow from their niche in high serum media (Pasut et al., 2013; Goetsch et al., 2015). The activated satellite cells (myoblasts) that migrate out of myofibers can be sub-cultured on the Collagen or Matrigel coated dishes. Therefore, a combination of Matrigel coating and use of high serum media that promotes migration of myogenic progenitors from myofibers onto the culture dish could be used to isolate activated satellite cells. This joint approach is shown to provide growth advantage to myoblast over other non-myogenic cells and maintain their myogenic differentiation capacity to fuse into myotubes *in vitro* (Shahini et al., 2018). The efficiency of this method is demonstrated to be high due to high number of satellite cells released from the skeletal muscle niche. The protocol described below used the combination of Matrigel coating with culture medium containing high serum, chicken embryo extract (CEE) and bFGF to isolate myoblasts by promoting the proliferation and migration of satellite cells out of their niche.

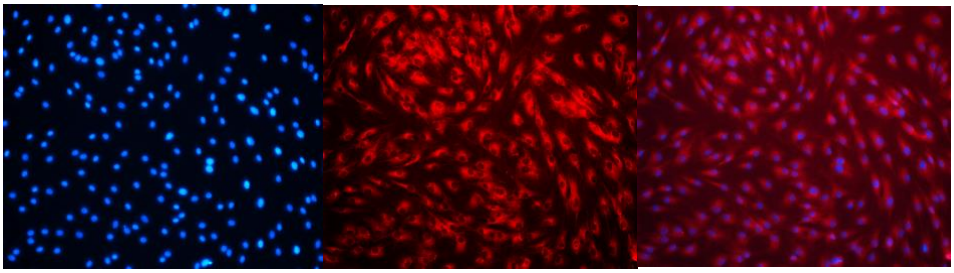
1. Skeletal muscles were harvested from the hindlimbs of nine-month old DMD^{mdx} and normal rats and fat tissue were discarded, and the muscle tissue was minced to small pieces and were transferred to a 50ml conical tube.
2. The small pieces of tissue were digested in 1ml of mixed Collagenase II, Collagenase D, and dispase II solution and incubated for 60 min in 37^{o}C water bath while agitating the tube every 5 mins.
3. The tube was centrifuged at 300xg for 5 min and the pellet was resuspended in the proliferation medium.
4. The suspension containing small pieces of muscle tissue was seeded on Matrigel coated plate at 10- 20% surface coverage and incubated at 37^{o}C to allow attachment of the tissues to the surface and subsequent migration of cells.
5. The cells were observed every two days by gently removing the flasks under phase microscope and new media changed to replenish growth factors.
6. Once the local cell confluency was observed, the muscle tissues were transferred to the new flask.

To further characterize the outgrowth cell population, immunocytochemistry (ICC) analysis of cytoskeletal myogenic marker of Desmin (Abcam1#5200) (Figure 3.1.B) and fibroblast marker of alpha smooth muscle Actin (α-SMA) (Abcam #5694) (Figure 3.2.B) were performed. Results showed that very small number of outgrowth cells from skeletal muscle tissue of 9-month old DMD ^{mdx} rat were myogenic progenitors. The majority of isolated cells using this method were fibroblasts as shown in the Figure 3.1, necessitating further purification steps by applying several rounds of pre-platting, fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS).



A. B. C.

Fig. 3.1: Immunofluorescence microscopy (10X) of outgrowth cells in DMD ^{mdx} rat cell culture
A) nuclear straining using DAPI (blue), B) anti Desmin antibody staining (red), C) DAPI and Desmin antibody staining merged



A. B. C.

Fig. 3.2: Immunofluorescence microscopy (10X) outgrowth cells in DMD ^{mdx} rat cell culture
A) nuclear straining with DAPI (blue), B) anti α-SMA antibody staining (red), C) DAPI and α-SMA Merged

3.1.3. Myoblast Isolation Protocol (3)

To yield high numbers of myogenic cells, bilateral lower extremity skeletal muscles including Extensor Digitorum Longus, Tibialis Anterior, Gastrocnemius, Soleus, Hamstring, and Quadriceps were harvested from two one-month-old DMD^{mdx} and littermate WT rats. The protocol was developed and previously used by Dr. Alec Smith, at University of Washington Department of Physiology and Biophysics (Smith et al., 2012). The details of dissociation and isolation steps are depicted in the Figure 3.3.

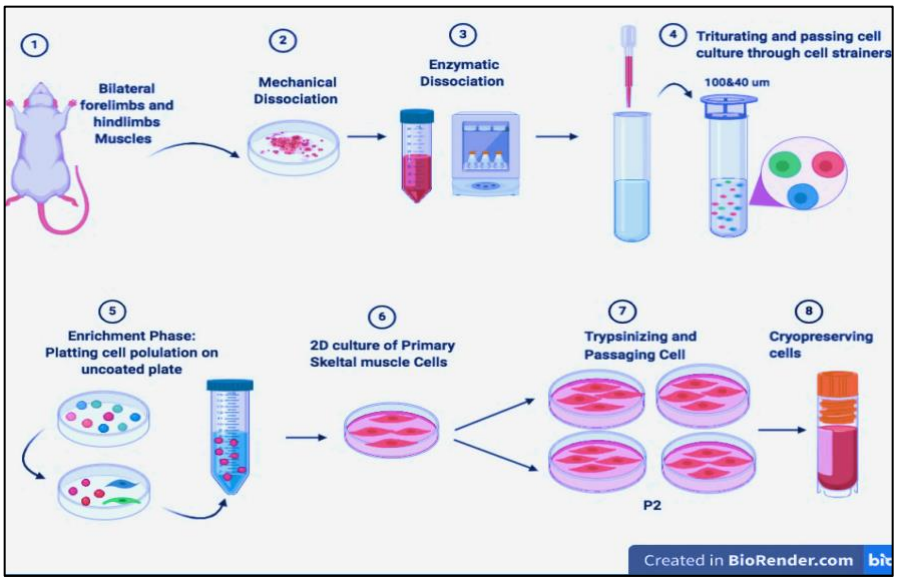


Fig. 3.3: Skeletal muscle harvest and isolation (Protocol #3)

1. Bilateral lower extremity muscles including Extensor Digitorum Longus (EDL), Tibialis Anterior (TA), Gastrocnemius, Soleus, Biceps Femoris, and Quadriceps of one-month old DMD ^{mdx} and its littermate Wild type rats were harvested separately.
2. Harvested tissue fragments were mechanically dissociated using the P1000 tip and then transferred to 15ml Falcon tube.
3. Supernatant was removed after centrifuge and 0.1% Collagenase was added (Gibco, cat # 17101-015). The tube was shaken vigorously to dissociate the fragments and then was transferred to a shaking incubator (at 37^{o}C) for 20-25 minutes.
4. The tissue was removed from the incubator and further triturated vigorously using a P1000 tip until the fragments were completely dissociated.
5. Cell suspension was passed through a 100 µm and then a 40 µm cell strainer to remove excess connective tissue and remaining debris
7. Myoblast were further enriched by pre-platting isolated cells that contained mixed cell population on uncoated plasticware for 1 hour. This enrichment steps caused differential

attachment of fibroblasts and other non-myogenic progenitors to the plate and separated them from myoblasts. Figure 3.4.A and 3.4.B demonstrated the morphology of non-myogenic cell pollution that were proliferated over 7 days following the enrichment step.

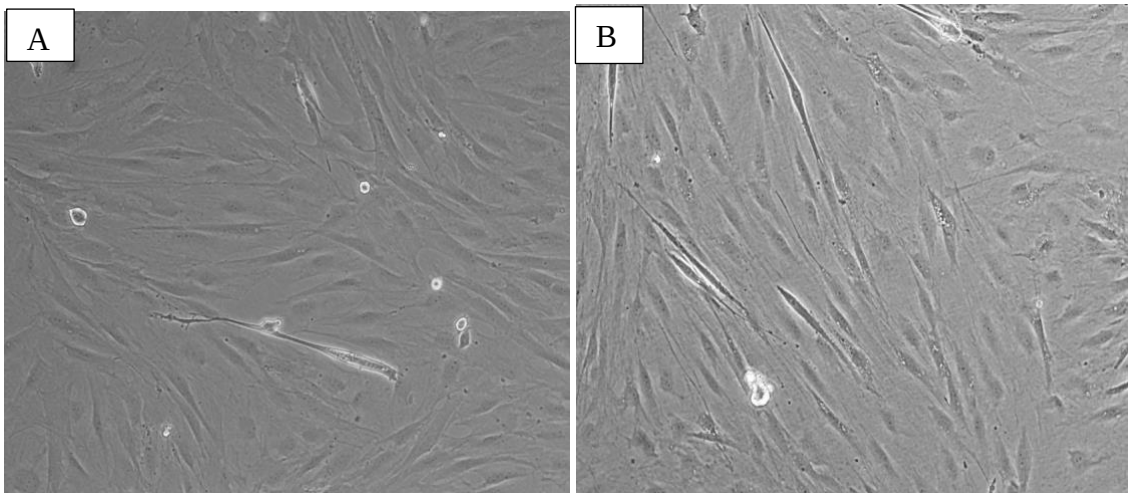


Fig. 3.4. Phase contrast microscopy (10X) of non-myogenic cells proliferated on day 7 post enrichment; A) WT, B) DMD

This protocol resulted in a high yield of $4x10^6$ cells from the bilateral lower extremity muscles of each rat. Isolated cell population were characterized with immunocytochemistry (ICC) analysis of myogenic cell markers such as Desmin and Myogenin (Figure 3.5). Primary antibodies selected for ICC analysis were Rabbit anti Desmin antibody (Abcam1# 5200) and Mouse anti Myogenin Antibody (Abcam #5694).

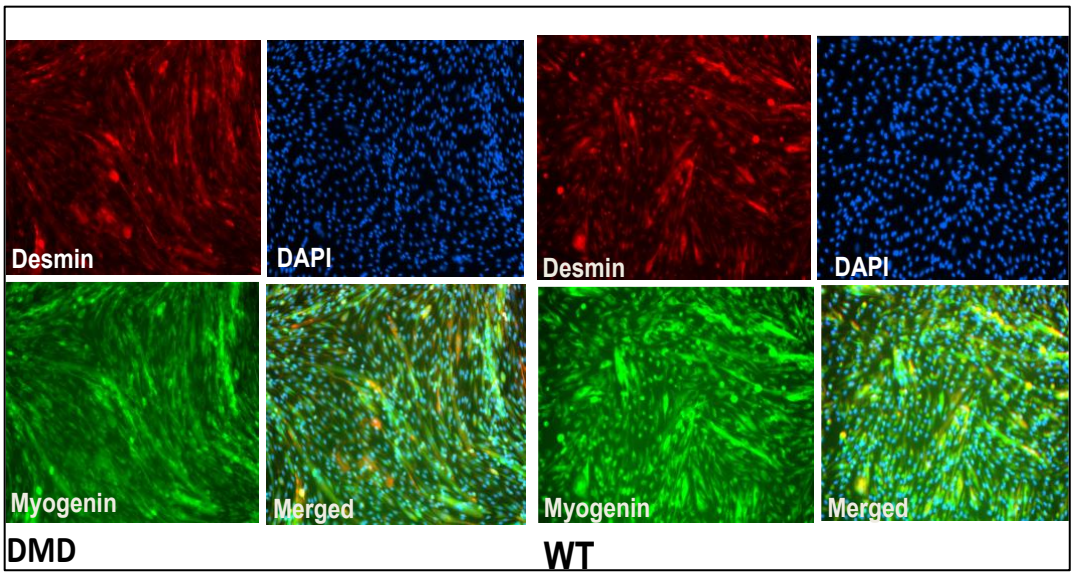


Fig. 3.5. Immunofluorescence microscopy (10X) of differentiated WT and DMD rat myofibers; Nuclear straining with DAPI (blue), Desmin staining (red), Myogenin staining (green) Merged DAPI, Desmin, and Myogenin stainings. Scale bar = 50um

3.2 Primary Myoblasts Growth and Expansion

Immediately following the enrichment step, collected supernatants from the uncoated (35 mm x10 mm) plastic plate that contained single cells suspended in growth media were seeded on Matrigel (Lot #7331010) coated plastic plate for further expansion. Matrigel at 9mg/ml was diluted in the Dulbecco's Modified Eagle Medium F12 (DMEM/F12) to a working concentration of 0.09 mg/ml, then diluted Matrigel solution were added to the plate at 0.05ml/cm^2 (45ug/cm^2). The myoblasts were plated at the recommended density of < 50% confluency in the Ham's F-10 Nutrient Mixture (F-10) medium (Gibco #11550043) supplemented with 20% Fetal Bovine Serum (FBS), 20 ug/ml bFGF, 1% penicillin/streptomycin and maintained at 37℃ in a humidified atmosphere of 5% CO_2 incubator. Proliferation medium was changed every day. Once the myoblasts proliferated and reached at 50% confluency (Figure 3.6. A&B), they were harvested from the plate using 0.05% Trypsin EDTA (Gibco #25200056), passaged for further expansion, and cryopreserved subsequently for future experiments.

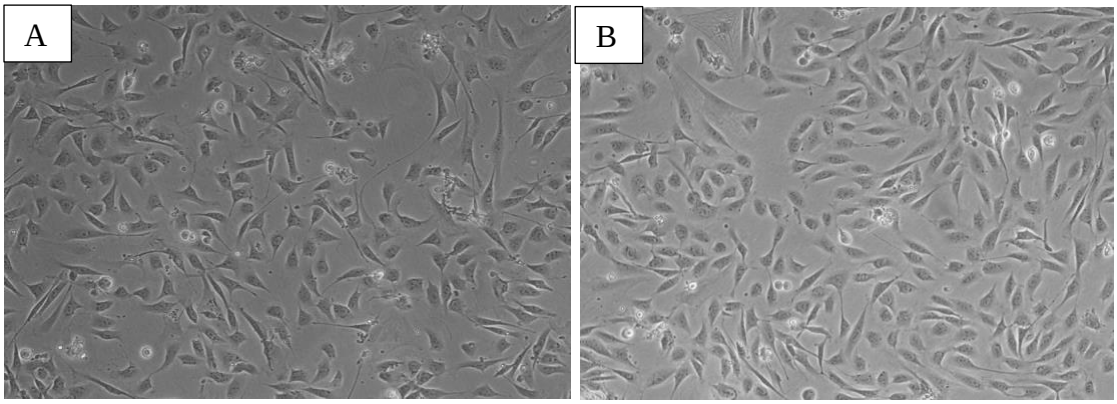


Fig. 3.6. Phase contrast microscopy (10x) of myoblasts proliferation on day one post platting; A) WT B) DMD

3.3 Applied Mechanical Stimulation (AMS) Platform

In vitro skeletal muscle development has significant role in the study of pathophysiology of neuromuscular disease, development of potential therapies for muscular disorders, and *in vivo* transplantation of cell therapies for aged, diseased and injured skeletal muscles. Optimized *in vitro* skeletal muscle model systems that recapitulate *in vivo* conditions and allow for accurate control of the experimental environment are highly desirable for detailed study of the molecular mechanisms of skeletal muscle adaptation and development. Design of *in vitro* skeletal muscle model system has been particularly challenging due to a complex cellular organization and electromechanical function of the tissue. Successful engineering of highly functional skeletal muscles may require creation of biomimetic culture conditions involving medium perfusion, electrical and mechanical stimulation. When optimized, these external cues are expected to synergistically and dynamically activate important intracellular signaling pathways leading to muscle growth and development.

Cell Stretching as a form of mechanical stimulation has been used extensively in *in vitro* models of skeletal muscle tissue engineering and regeneration (Rangarajan, et al., 2014). Physical input in the form of mechanical stimulation have a longstanding track record in rehabilitation medicine to promote the recovery of muscle function after injury and possibly delay disease progression. Artificial application of this inductive stimulus in a dish will mimic static/dynamic stretch in the embryo. Static and cyclic mechanical loading affects muscle cell growth and differentiation through mechanotransduction pathways leading to the up-regulation and secretion of autocrine and paracrine factors, including insulin-like growth factor 1 (IGF-1), ATP, reactive oxygen species (ROS) and prostaglandins. In striated muscle cells, these molecules will activate downstream effectors like phosphoinositide 3-kinase (PI3K), G-proteins, mitogen-activated protein kinase (MAPK) and AMP-activated kinase (AMPK). The coordinated activity of these signals in response to mechanical strain leads to up-regulation of muscle-specific transcripts, cell growth and hypertrophy (Hornberger & Esser, 2004; Hornberger et al., 2005; Martineau & Gardiner, 2001; Musi & Goodyear, 2003; Cheema et al., 2005; Goldspink et al., 1992).

In animal models, previous studies of avian and rodent skeletal and cardiac muscle cells showed improvement of orientation, density, and contractile function of myofibers following static or intermittent cyclic stretch (Collinsworth et al., 2000). At the molecular level, a faster progression of the myogenic gene expression is evident as myogenic determination markers MyoD and Myogenin significantly upregulated when strain is applied (Liao et al., 2008; Candiani et al., 2010; Heher et al., 2015). After mechanical stimulation, human cardiomyocytes and/or skeletal muscle cells showed signs of increased proliferation (Tulloch et al., 2011), myofiber diameter (Powell et al., 2002), density, tensile resistance and contractility (Akhyari et al., 2002; Moon et al., 2008). However, not all regimes of mechanical stimulation have positive effects on striated

muscle differentiation. Boonen et al. showed a decrease in myogenic regulatory factors and a delay in sarcomerogenesis upon application of 2% uniaxial ramp stretch followed by 4% uniaxial intermittent dynamic stretch in C2C12 cells (Boonen et al., 2010). Similarly, specific regimes of biaxial stretch inhibited myogenesis in favor of continued myoblast proliferation or delayed satellite cell activation (Kumar et al., 2004; Otis et al., 2005). Table 3.1 summarizes the positive effects of mechanical stimulation with varying regimens on cardiac and striated muscle cells.

Table 3.1. Summary of *in vitro* cardiac and skeletal muscle studies of mechanical stimulation

Study	**Cell Type**	**Type of stimulation**	**Rate of Stimulation**	**Duration**	**Frequency**	**Outcome**
Powell et al., (2002)	HSMC	Mechanical	5% (2days), 10% (2days), 15% (4 days) Cyclic	8 days	0.5 Hz	Increased myofiber diameter, density
Akhyari et al., (2004)	Pediatric CMs	Mechanical	20 % Cyclic Stretch	14 days	1.34 Hz	Improved CM proliferation and mechanical Strength
Moon et al., (2008)	HSCM	Mechanical	10 % Cyclic Stretch	5-21 days	0.05 Hz	Improved force generation post implantation
Tulloch et al., (2011)	Human ESC/iPSC-CMS	Mechanical	5 % Cyclic Stretch	4 days	1 Hz	Increased CM proliferation & hypertrophy
Kensah et al., (2013)	Mouse & Human iPSC-CMs	Mechanical	10% Cyclic, 0 -23 % Static Ramp Stretch with 2% every 2nd day	7 days 14 days	1 Hz	Improved Cardiac Tissue formation
Mihic et al., (2014)	HSCM	Mechanical	12 % Cyclic Stretch	14 days	1.25 Hz	Increased contractile function, increased cell elongation

In vitro AMS model system requires finely optimized components leading to optimal skeletal muscle growth and differentiation. These components are; 1) AMS parameters, 2) substrates topography, 3) cell culture matrix, and 4) temporal induction of biochemical compounds. Collectively, these factors affect the ultimate adaptive responses of cultured cells to applied mechanical stimulation and their maturation into functional skeletal muscle models. The following sections explain in detail the optimization efforts to develop passive-stretch induced skeletal muscle injury platform.

3.3.1 Optimization of AMS System

The correlation between mechanical force application and genotypic/phenotypic cellular responses has prompted numerous studies in recent years to investigate the optimal cues that promote tissue maturation. In this pursuit, different research groups have developed platforms that artificially apply mechanical force to tissue cultures through the application of uniaxial stretch (Iwata et al., 2009; Passey et al., 2011; Kasper et al., 2018). Different patterns of mechanical stimuli are defined as single stretch regimes that consist of numerous highly modifiable parameters. In general, six parameters are essential when designing stretch experiments. These parameters are listed below:

1. Stretch ratio = % pull of resting length.
2. Frequency = number of cycles per second.
3. Duration = length of study.
4. Rate = stretch length per time.
5. Optimal start day = when myoblasts are deemed mature enough to perturb efficaciously.
6. Rest period = whether this be a full rest with no stretch or a recovery period with reduced stretch.

While, these parameters have been tested to better understand the cellular mechanism of mechanotransduction (Kasper et al., 2018), the optimization of these AMS parameters has not yet been fully explored. One complication in the pursuit of optimizing AMS parameters is the lack of standardized stretch application techniques. Current experimental designs for implementing AMS vary widely in terms of the systems and protocols that are used. Some groups have created custom

cell stretching devices to conduct stretching experiments (Vandenburgh et al., 1989; Heher et, al., 2015) while others have used commercially available cell-stretching devices such as Strex™ and Flexcell ™. However, each of these devices have shortcomings that could potentially limit the generalizability of results to future experimentation.

The Strex™ system consists of highly versatile standard cell stretching system, which is capable of equally stretching cells to cultured cells in ultra-thin flexible PDMS stretch chambers. It simultaneously stretches cells in multiple chambers to enable comparison between samples. It has several other unique features such as 1) providing uniform strain along the stretch axis, 2) high reproducibility, precision, and torque stepping motor in the stretch unit, enabling stable motion at a range of speeds from low to high velocity, 3) wide range of stretch patterns, providing eight different settings for degree of stretch ratio and repetition frequency of stretch that makes for 64 possible stretching. However, the limited options for stretch application provided by Strex system limit device application. In fact, physiologically relevant stretch parameter settings could possibly lie in between the available 64 options, but system limitations would prevent exploration of these potential optimizations. Moreover, another limitation of the system is that it only allows testing of stretching parameters up to 20% stretch. Additionally, there is a limitation on the number of chambers that could be tested simultaneously in the stretch unit. Two versions of the main stretch unit are available, depending on the size of the stretch chamber; 4 cm^2 or 10 cm^2. The smaller 4 cm^2 model supports up to 8 chambers in parallel, whereas the 10 cm^2 version supports up to 6 chambers. This puts restriction on the number of chambers that can be simultaneously examined in any given experiments.

One of the main complications we ran into was the quality of primary skeletal muscle cell growth and maturation on the flat surfaces using the Strex™ Cell System culture ware. The flat

culture ware required extensive optimization of ECM proteins coating on the nanopatterned chamber to better control the quality of myofibers differentiation and maturation.

A novel cell stretching system is currently being developed to overcome the deficiencies found with the existing selection of cell-stretching devices. The Seattle-based Biomedical company, Curibio^{R}, designed a prototype device called the Cytostretcher^{TM} that was close to commercial availability in 2019. The Cytostretcher^{TM} system consists of three primary components: the mechanical stretching device, accompanying custom cultureware, and dedicated software called the NanoSurface Operational Mechanics Interface (NaOMI) (Figure 3.7). The custom-made culture ware comprises a selection of differently sized flexible Polydimethylsiloxane (PDMS) chambers (Figure. 3.8). Cells are grown inside these chambers which are easily loaded into the mechanical stretching device, and the device is small enough to be placed inside a standard laboratory incubator. Stretch regimens are programmed using the NaOMI software: an intuitive interface that enables fast creation of unique, complex, and precise force application patterns in limitless ways. The software is initially run from a computer, but the created regimen can be sent to an external controller on the device that will run the regimen without requiring computer connection. The 6 primary stretch parameters identified earlier are easy to program and allow recreation and expansion of experimental conditions used by other research groups.

Fig. 3.7 Cytostretcher cell stretching system

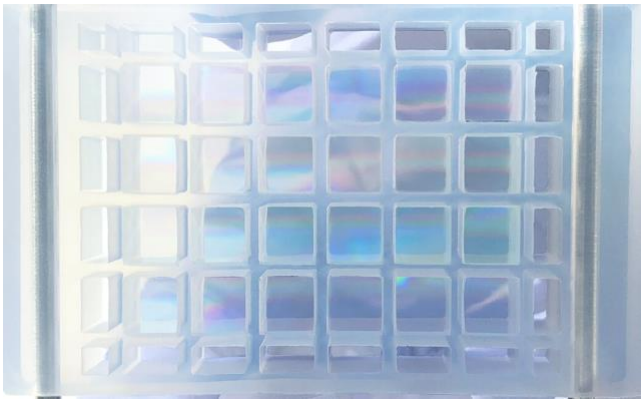


Fig. 3.8 Cytostretcher 24-well plate chamber

The Mack lab has been interested in studying the effects of mechanical stimulation on skeletal muscle development and maturation in normal and diseased conditions. The Cytostretcher cell stretching system is an integrated platform for creating mechanically conditioned cell and tissue models. The system allows researchers to investigate both tissue-level mechanical strain and microenvironmental cues at the same time. One unique feature of the system is the Nanotopography patterning technology which provides structural cues that recapitulate the native ECM within flexible stretching chambers. Moreover, it provides a uniform physiologic environment to best examine the response of cultured cell aligned to the direction of stretch.

The Mack lab was the first lab that initiated a research agreement with Curibio^{R} to test the Cytostretcher^{TM} in exchange for the opportunity to perform early experiments with the device before official market release in 2019. However, at the beginning of this project in 2019, the Cytostretcher^{TM} system was still in a prototype stage and had multiple issues hindering proper functionality. Therefore, one important aspect of this project was to assist the company troubleshooting all components of the system. In close collaboration with Curibio^{R}, discovered issues were communicated to facilitate early commercial distribution. Besides, the standard laboratory procedures concerning device use and cell culture technique in the custom Cytostretcher^{TM} cultureware were optimized to ensure best results for future experiments. Here, results of troubleshooting of Cytostretcher^{TM} prototype is presented:

The programming interface of the Cytostretcher^{TM} software, NaOMI, initially had issues maintaining connection to the Cytostretcher^{TM} device which prevented running stretch regimens entirely. Connecting the device to NaOMI normally consists of simply connecting a USB cable from the external controller of the device to a port of a computer running the software then pressing connect on the interface. However, in practice the software often displayed that the

device either failed to connect, could not connect, or that the USB device was not recognized. Naomi software often failed to connect to the device during preliminary tests. Device connection was mainly impacted by disconnecting and reconnecting the Cytostretcher^{TM} device from the power source. The ability of the software to measure progress of the defined protocols was limited to only tracking stretch time, frequency, and number of cycles. In the initial version of NaOMI , stretch length was not included in this log, which limits troubleshooting capabilities.

Multiple software issues prevented AMS designs programmed in NaOMI from being implemented as expected by the Cytostretcher^{TM} device. Normally, stretch regimens can be generated by putting together independent segments occurring over defined times where stretch ratio, frequency, rate, duration, and rest period are defined by inputs to several parameters. The NaOMI user interface has two important tools called “duplicate segment” and “repeat”. There are several user-interface tools available to expedite this programming (Figure 3.9). The “duplicate segment” tool copies an existing segment and reproduces an identical, separate segment to avoid needing to reenter stretch parameters again.

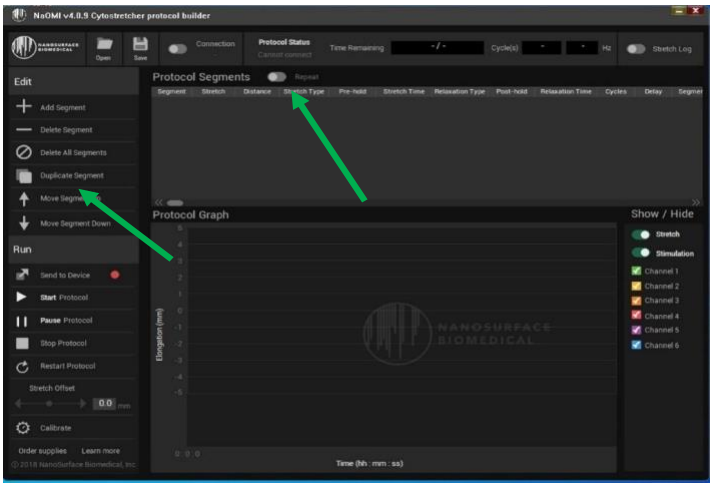


Fig.3.9. NaOMI software (version 4.0.9) user interface

This is useful for many applications because both segment copies can be moved independently throughout the protocol to create patterned regimens. The “repeat” toggle switch infinitely loops through all the defined protocol segments enabling long protocol run times without needlessly recreating every segment in the file multiple times. While intended to expedite programming, these user-interface tools caused disruptions in the software. Use of the duplicate segment tool prevented the ability to save and reopen the current protocol file. Alternatively, protocols that were created using the duplicate segment tool and ran immediately without saving would spontaneously and randomly terminate. Protocol termination meant the Cytostretcher™ motor was fixed at a random position between the baseline and maximum stretch length while the software also displayed the fixed length. The program timer still tracked the elapsed time from when the protocol began indicating the software was not frozen, but the motor would not move again until the current protocol stopped. This issue occurred regardless of whether the protocol was running directly from the software or the external controller. To run experiments so that the programmed regimen could be implemented by the stretcher without issue, stretch protocols needed to be created manually; that is, without assistance from the duplicate segment tool.

Additionally, when the protocol was sent to the external controller with the repeat protocol segment option toggled on, the external controller would not execute the command and only run the complete protocol once. To utilize the repeat function, the stretch protocol needed to be run from a computer instead of the external controller. To run a protocol intended to repeat from the external controller without requiring a computer, repeated segments needed to be entered manually before sending to the controller. In summary, activation of “Duplicate segments” caused random stop of protocols, disruption of execution time and “repeat” function produced invisible extra second delay in execution and increased protocol time. Therefore, we chose not to use duplicate

segment function to prevent the unintended disruption of protocol.

3.3.2. Optimization of Cell Culture Environment

Nanotechnology has recently been used to promote structural and phenotypic development by aligning cells with nanoscale topography. Skeletal and cardiac muscle cultures maintained on nanoscale topographies show enhanced cellular alignment over cells on un-patterned plastic (Yang et al., 2014; Carson et al., 2016). Furthermore, cells cultured on nanosurface display an enhanced structural and phenotypic development and undergo maturation in terms of MyHC isoform expression, sarcomere development, and muscle regulatory factor expression profiles (Yang et al., 2016). Application of this technology represents a promising alternative for the development of more physiologically structured tissues, thus it could be used for diseases modeling in which lateral connectivity between muscle fibers and their surrounding matrix is impaired, such as muscular dystrophy.

Curibio^{R} offers flexible PDMS nanopatterned chambers that are compatible with Cytostretcher^{TM} device. The cell culture surface of each chamber is a flexible silicone-based sheet with a Young's modulus of 2-3 MPa which is modified to have 800 nm nanopatterned ridges parallel to the stretching axis. Nano topography provides an advantage over flat culture ware used in previously designed cell-stretching system, because in skeletal muscle, nanosclae topography promotes anisotropic myoblast alignment along the axis of stretch (Figure 3.10).

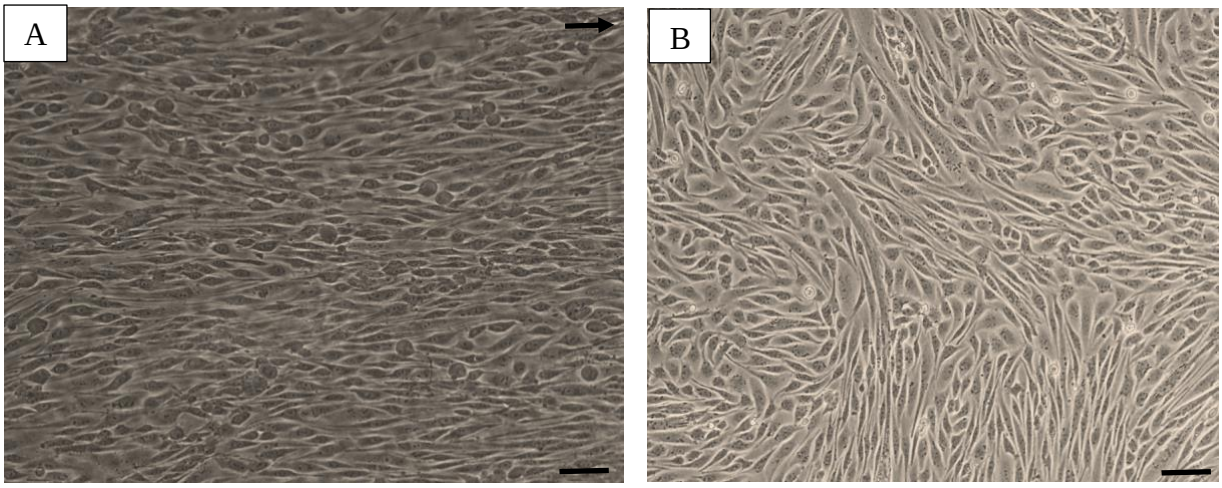


Fig. 3.10. A) Phase contrast microscopy (10X) of WT myoblasts proliferated on Nanosurface- (1 day post platting) B) myoblasts proliferated on plastic surface (one day post platting) (→ = Direction of nanopattern print, Scale bar = 100 um)

3.3.3. Optimization of Cell Culture Matrix

Skeletal muscle fibers reside in a three-dimensional scaffolding that consists of various collagens, glycoproteins, proteoglycans, and elastin, and is commonly referred to as extracellular matrix (ECM). ECM has multiple functions including cell adhesion, cell proliferation, cell differentiation, cell structural support, and tissue regeneration. These functions are carried out by the different cellular components within the ECM. A significant challenge in *in vitro* skeletal muscle tissue development is to recreate the proper biomimetic structural organization of the native muscle to ensure the efficient generation and transmission of contractile force. *In vivo*, interactions between the surface of myofibers and the basement membrane ensure the effective transfer of the contractile force (generated in the sarcomeres) laterally across the cell surface to ECM for full force generation (Csapo et al., 2020). One of the challenges in the face of proper skeletal muscle proliferation and maturation in this project was to maintain myogenic cells in differentiation phase long enough to be able to accurately examine the response of differentiated myofibers to stretching.

For the purpose of this project, various ECM proteins solution with different concentrations were tested and results are described below.

Cell cultures in PDMS nanopatterned surface requires preparative steps to ensure proper sterility and enhanced adherence of cultured cells to the chamber surface. Prior to coating chambers with ECM, chambers were first washed with 70% ethanol and allowed to air dry for 10-15 minutes. Once dry, the chambers placed under UV sterilization for 20 minutes and then were plasma treated for 5 minutes. Immediately after plasma treatment, the chambers were coated with Poly-D Lysin solution (PDL) for one hour to enhance cell attachment to surface. Then, PDL solution was removed and chambers were washed three times with distilled water and dried for 30 mins prior to ECM coating.

3.3.3.1 Matrigel

Matrigel matrix is a protein mixture extracted from mouse tumor, which is rich in ECM protein. It is composed of approximately 60% laminin, 30% collagen IV, and 8% entactin. It also contains heparan sulfate proteoglycans, that helps with cell attachment in synergy with integrins and other adhesion receptors, and a number of growth factors such as transforming growth factor beta (TGF-β), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). Matrigel has four integrin binding sites (α1β1, α2β1, α6β1, α7β1) and performed excellent in mouse and human myoblast cell cultures in short-term studies but showed high amounts of variability following long-term expansion (Hughes et al., 2010). Therefore, we examined long-term primary rat myoblast cell culture growth and differentiation on the nanopatterned Cytostretcher^{TM} chamber coated with Matrigel.

- Substrate: PDMS nanopatterned chambers pre-treatedwith plasma treatment and PDL prior to Matrigel coating, coated chambers incubated in the incubator overnight
- ECM: A growth-factor-reduced Matrigel (Corning # 356231)
- Platting Density: 50k cells per chamber (Size of chamber:12mmx12mm)
- Final Concentration:150 ug/ml (1:60 dilution ratio)
- Proliferation Media: Ham's F10 Nutrient Mix, 20% FBS, and 20ug/ml bFGF
- Proliferation media was changed every day
- Differentiation Media: DMEM (High Glucose) media, 2% Horse Serum (HS), and 20 ug/ml IGF
- Differentiation media was changed every other day

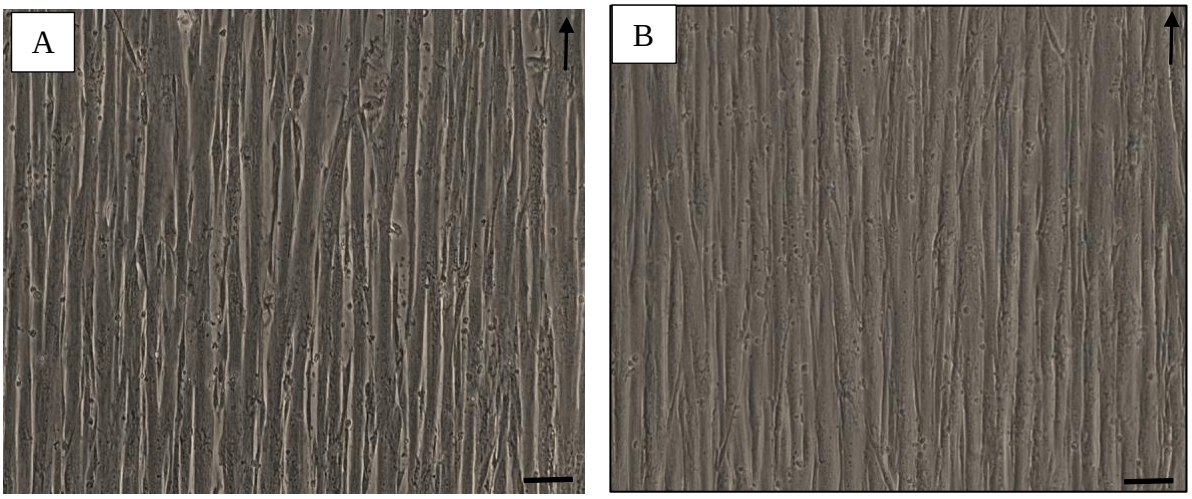


Fig. 3.11. Confocal Microscopy (10X) of myofibers at day four post differentiation: A) DMD, B) WT (Scale bar = 100 um)

Myoblasts coated on PDMS nanopatterned surfaces using Matrigel observed to proliferate similarly in both cell line. Myotubes differentiated to elongated myofibers very well on the Matrigel substrate and WT myofibers look more hypertrophic, compared to DMD myofibers (Figure 3.11). On day 3 post differentiation, the myofibers in both cell lines started to detach from the surfaces. This finding suggested that Matrigel supports primary rat WT and DMD ^{mdx} myoblast proliferation; however, long-term survival of developing myofibers was not well maintained.

3.3.3.2 Laminin 521

Laminin serves as ligand for two sarcolemma receptors- the dystrophin-associated glycoprotein complex and the α7β1– located at costameres, which are membrane-bound protein structures aligned in register with the Z-disks of myofibrils. Laminin 521 is a xeno-free cell culture matrix that supports the single-cell attachment and long-term maintenance of mouse and human myoblast cell cultures. Laminin 521 supported increased proliferation in early phases of expansion and was the only substrate facilitating high-level fusion following eight passages in mouse myoblast cell cultures. In human myoblast cell cultures, laminin 521 supported increased proliferation during expansion and superior differentiation with myotube hypertrophy (Penton et al., 2016). This advantage of laminin 521 are likely due to the larger number of integrin binding sights on laminin α5 including unique binding sites for integrin α3, αV, and α6β4 that are not present in other laminin isoforms (Hirosaki et al., 2000). Thus, Laminin 521 can be perceived as a more potent substrate capable of interacting with six integrin binding sites promoting cell adhesion to the surface. However, it has not been tested in WT and DMD ^{mdx} primary rat myoblast cell cultures. Therefore, we evaluated long-term primary rat skeletal muscle cell culture growth and differentiation on the nanopatterned Cytostretcher^{TM} chamber using Laminin 521 coating. To do this, we performed the following steps:

- Substrate: PDMS nanopatterned chamber pre-treated with the plasma treatment and PDL prior to Laminin 521 coating, coated chambers kept in the incubator overnight
- Platting Density: 50k per chamber (Size of chamber: 12mm x12mm)

- Final concentration of Laminin 521(STEM CELL # 77003); three different concentrations of laminin 521 were tested in separate experiments, 10 ug/ml, 20 ug/ml, and 50 ug/ml incubated in the incubator overnight
- Proliferation Media: Ham's F10 Nutrient Mix media, 20% FBS, 20 ug/ml FGF
- Proliferation media was changed every day
- Differentiation media: DMEM (High glucose) media, 2% Horse Serum, 20 ug/ml IGF Differentiation media was changed every other day.

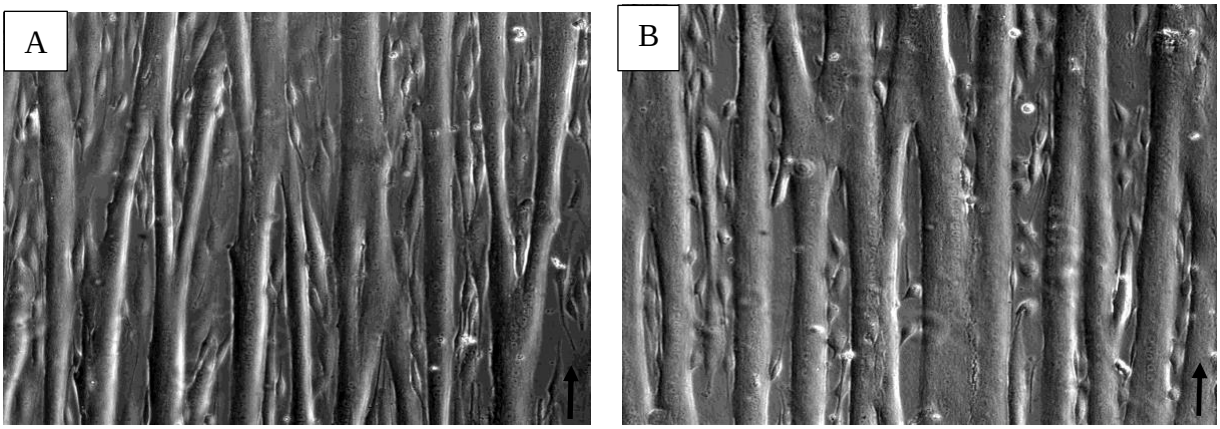


Fig.3.12. Confocal Microoscopy (20X) of myofibers on day four post differentiation, Laminin-521(10 ug/ml); A) WT, B) DMD

Myoblasts platted on PDMS nanopatterned surfaces coated with Laminin 521 (10 ug/ml) proliferated very well similarly in both cell lines. Myotubes differentiated to elongated multinucleated myofibers and myofibers in DMD cell line appeared to be more hypertrophic compared to WT cell line (Figure 3.12). However, on day 5 post differentiation, myofibers in both cell lines detached from the surfaces. Same results observed with the higher concentrations of Laminin 521 (20 ug/ml & 50 ug/ml). This finding suggested that laminin 521 supports primary rat myoblast cell culture proliferation, however long-term survival of developing myofibers was not preserved. For the subsequent experiments, substrate coating using 150 ug/ml Matrigel along with

additional supplement of differentiation media with Matrigel, i.e. Matrigel Sandwitch, to further stabilize the cell culture in order to inhibit myotubes detachment was pursued.

3.3.4 Optimization of Differentiation Induction

To control maximal efficiency of skeletal muscle growth and differentiation *in vitro*, biochemical cues to developing myotubes are required. Activation of signaling pathways like Wnt, Notch, IGF, and MAPKs have implications in different stages of *in vitro* myogenic progression. Wnt and Notch can be manipulated by early introduction of cultured myoblast or differentiating myotubes to exogenous biochemical molecules. Wnt signaling's primary role during *in vitro* myogenesis is myoblast differentiation and myotubes fusion (Jones et al., 2015). Therefore, exogenous expression of Wnt using biochemical material is shown to be effective in driving the myogenic lineage *in vitro*.

To further improve muscle functions, the activation of cell-signaling pathways from myogenic growth factors, such as IGF-I, is critical. IGF-1 is a member of insulin-related bioactive peptides that is produced locally by skeletal muscle and liver cells in the paracrine and autocrine fashions, respectively. In skeletal muscle tissue, IGF-1 is highly upregulated upon differentiation, inducing the expression of differentiation markers in satellite cells. In a similar way, in later stages of skeletal muscle differentiation, IGF-1 is highly upregulated upon differentiation of myotubes, inducing the expression of differentiation myogenic markers like Myogenin and also is capable of activating multiple MAPKs (Dai et al., 2010). Thus, exogenous introduction of IGF-1 molecule has significant implication in the terminal differentiation of skeletal muscle cells *in vitro.*

Skeletal muscle development involves an initial period of myoblast proliferation, followed by a phase in which some myoblasts continue to proliferate while others undergo terminal differentiation. The latter process involves the cessation of DNA synthesis and activation of

muscle-specific gene expression, and the fusion of single cells into multinucleated muscle fibers. *In vitro* studies indicate that the onset of terminal differentiation is delayed by media that are rich in serum and growth factors like FGF. When exponentially growing myoblasts are deprived of FGF, cells become postmitotic within 2-3 hours, express muscle-specific proteins within 6-7 hour, and begin fusion within 12-14 hour. Although expression of these three terminal differentiation phenotypes occurs at different times, all are initiated by a single regulatory commitment event in G1. Eventually, the entire population commits to terminal differentiation with in 12 hours of FGF removal as cells complete the cell cycle and move into G1(Clegg et al., 1987).

During myoblast proliferation, primary myoblasts are grown in proliferation media that contains 20% Fetal Bovine Serum (FBS), F10 Ham's Nutrient Mix, and FGF (20 ug/ml). Proliferating myoblasts are highly proliferative, because FBS contains factors for proliferation. In order to differentiate myoblasts to myotubes, cell culture media needs to be withdrawn from proliferation and be switched to differentiation state. This is accomplished by supplementing the medium with adult Horse Serum (HS) instead of FBS. Lower percentage of horse serum (2%) ensures that proliferation is slowed down considerably (Andrés & Walsh, 1996). Therefore, we examined the differentiation media induction method explained above to assess the quality of myofibers differentiation on the nanopatterned Cytostretcher^{TM} chamber. To do this, we performed the following steps:

- Substrate: PDMS Nanopatterned chambers were pre-treated with plasma treatment and PDL, followed by Matrigel coating, and coated chambers were incubated in the incubator overnight.
- ECM: A growth-factor-reduced Matrigel (Corning # 356231)
- Platting Density: 50k cells per chamber (Size of chamber:12mmx12mm)

- Final Concentration:150 ug/ml
- Proliferation Media: Ham's F10 Nutrient Mix, 20% FBS, and 20ug/ml bFGF
- Proliferation media was changed every day
- Differentiation media: DMEM (High Glucose) media, 2% Horse Serum, 20 ug/ml IGF, Matrigel Sandwich (1:30 dilution ratio)
- Differentiation induction was started with 2% Horse Serum on day of differentiation induction, followed by change of same media every other day.

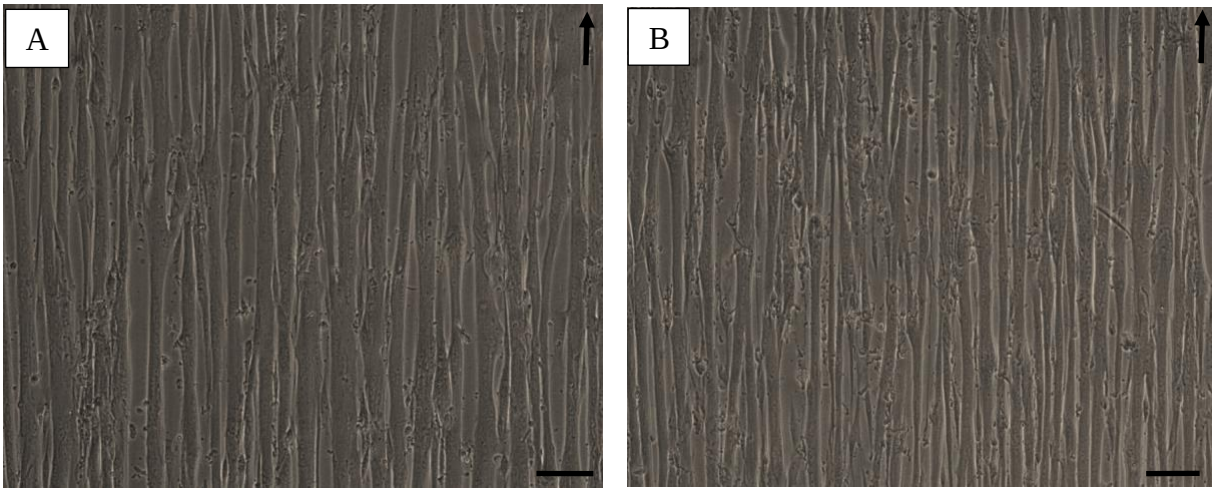


Fig. 3.13. Confocal microscopy (10X) of differentiated myofibers; day four post differentiation; Scale bar= 100 um A) WT B) DMD

Both WT and DMD ^{mdx} myoblasts differentiated into elongated multinucleated myofibers along the direction of nanopattern. Differentiated myofibers stayed attached to the substrate for four days post differentiation (Figure 3.13). Although Matrigel sandwich technique used on day of differentiation induction partially facilitated an inhibition of myofibers' detachment, it did not resolve the issue completely. During WT and DMD ^{mdx} rat primary myoblasts cell culture, it was noticed that differentiating myotubes started contracting spontaneously as soon as day two of differentiation. This observation highlighted high myogenic differentiation capacity as evidenced by formation of multinucleated myotubes capable of spontaneous beating. In fact, active force

generated by spontaneous contraction of myotubes as they differentiate and become mature myofibers might explain the underlying reason for the early myofibers detachment. This observation was more prominent in WT myofibers compared to DMD.

In an effort to overcome this shortcoming and further prolong myofibers' survival, "pre-stretching" technique wherein platted myoblasts were passively stretched using the Cytostretcher device was tested in series of five experimental design. This technique was initially designed to decrease substrate stiffness and make cell culture conditioned to passive tension before myoblasts start to differentiation. This technique was demonstrated not to make noticeable difference in terms of overall cell culture survival. Therefore, we tested the effect of low dose 2,3-Butanedione monoxime (BDM) to inhibit active contraction of myotubes during differentiation.

3.3.5 Optimization of BDM dose

BDM is well-characterized, non-competitive inhibitor of skeletal muscle myosin-II (Sellin et al., 1994). The inhibitory mechanism of action of low BDM concentrations (< 2mM) is through a reduction of net amount of Ca^{2+} release from the sarcoplasmic reticulum. Higher concentration of BDM (>10mM) directly affects the contractile apparatus by decreasing the number of interacting cross-bridges between the myofilaments (Yarrow et al., 2003). We examined three concentrations of BDM, 0.5mM, 1mM and 2mM, to identify the optimal dose to inhibit spontaneous contraction. The findings suggested that 1mM BDM has the optimal inhibitory effect on inhibition of spontaneous contraction of myofibers (Figure 3.14). In the subsequent experiments, BDM was added at 1mM concentration to the differentiation media to control muscle contraction. We determined that as a result of inhibition of active force generation and consequent reduction of fatigue, this AMS system design provided the most desirable platform to examine acute response of normal and dystrophin-deficient skeletal myofibers to passive-stretched induced

injury. With all optimization steps completed using the above-described set-up and protocols, we felt confident to proceed with testing the hypothesis in the following experimental design.

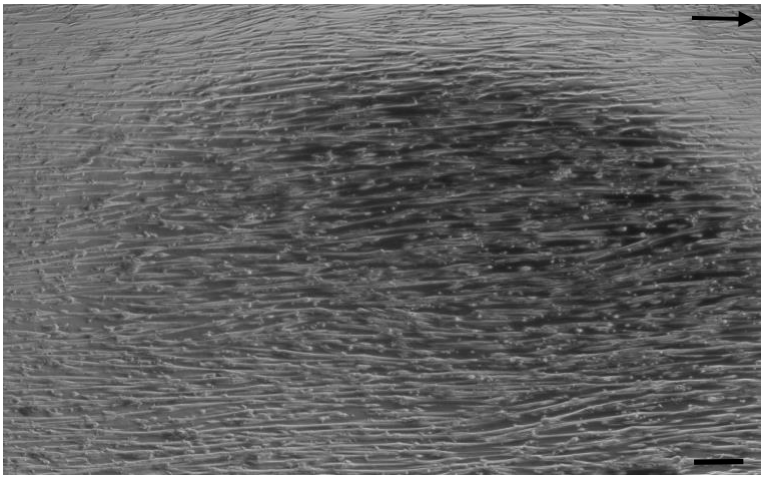


Fig 3.14. Bright field microscopy (4X) on day four post differentiation; WT Myofibers treated with 1mM BDM (Scale bar= 200um)

3.4 Passive-stretch Induced Injury

In the following sections, the methods used to prepare cell culture, Cytostretcher parameters configuration, and sample collection for electrochemiluminescence assays were explained in detail.

3.4.1 Cell Culture Preparation

WT and DMD^{mdx} myoblasts were plated on the 24-well plate Cytostretcher^{TM} nanopatterned chambers with initial platting density of 80% confluency. Half of the plate (12 chambers) was allotted for platting myoblasts in each cell line. In parallel, one 24-well plate Cytostretcher^{TM} nanopatterned chamber was platted with WT and DMD^{mdx} myoblasts as a control arm in the study. The proliferated myoblasts maintained in proliferation media until reached 100% confluency. Proliferation media consisted of Ham's F10 Nutrient Mix, 20% FBS, 20ug/ml FGF. Then, the proliferation media switched to differentiation media contained 2% Horse Serum, 1% of penicillin/streptomycin, Dulbecco's Modified Eagle Medium (DMEM) high glucose, and 20 ug/ml IGF. Myoblasts were allowed to differentiate into multinucleated myotubes for a 4-day period during which media was changed every other day. 20 ug/ml fresh IGF and 1mM BDM were added to the differentiation media every day to enhance myofibers differentiation and inhibit spontaneous contraction of differentiating myofibers. This experimental setting was replicated three times for future electrochemiluminescence.

In a parallel line of inquiry to test the second hypothesis, same experimental setting as described above was designed, with only additional step to transduce adeno-associated virus (AAV) to myoblasts on day of differentiation induction. Based on the recommendation of the

Chamberlain lab, AAV6-CK8-uDys5 vector (titer =1.6e^{13} vg/ml) was used. To best transduce the microdystrophin gene, multiplicity of infection (MOI) of 10,000 vg/cell was prepared for this purpose. AAV6-CK8-uDys5 was added to the differentiation media on day of differentiation induction overnight and the media containing vectors changed the next day.

3.4.2 Cytostretcher Configuration

AMS parameters were designed using the parameters provided by the NaOMI software version 4.0.9. These parameters include stretch ratio in millimeters, stretch type (sinusoidal or ramp length increase), pre-hold, stretch time, relaxation type (sinusoidal or ramp length decrease), post-hold, relaxation time, segment duration, and segment delay. Stretch type, pre-hold, and stretch time pertain to lengthening, while relaxation type, post-hold, and relaxation time pertain to shortening. Differentiated myofibers from the experimental plate were subjected to uniaxial stretch along the axis of chamber on day four of differentiation using the Cytostretcher^{TM} system. While the control arm, did not receive any stretch. Previous *in vitro* studies that replicated the *in vivo* eccentric exercise or passive stretch physiologic response such as increased hypertrophy and/or Creatine kinase release were used as reference for selection of stretch parameters (Passey et al., 2011). The parameters of stretching protocol selected on the Naomi software were as below (Figure 3.15):

Stretch ratio: 20% strain relative to muscle fiber length/length of chamber
Stretch distance: 1.2 mm elongation on 24-well plate chambers
Pre-hold: 0 second
Stretch Type: Sine wave
Stretch Duration: 30 seconds
Hold: 1 min
Relaxation Type: Sine wave
Relaxation Duration: 30 seconds

Post-hold: 0 second
Duration: 10 hours

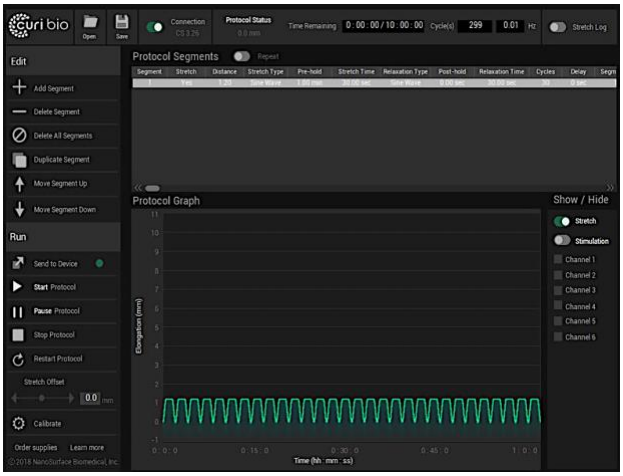


Fig.3.15. NaOMI passive-stretch parameters

3.5 Electrochemiluminescence Assay

In this project, Meso Scale Discovery^{R} (MSD) multi-spot Assay system (Meso Scale Discovery, Gaithersburg, MD, USA) was used to measure concentration of proteins of choice following stretch-induced skeletal muscle injury. Sample of differentiation media (25 ul) from both experimental and control plates were collected on Day 3 post differentiation. Media sample collection was repeated on Day four post differentiation from the same chambers in both control and experimental plates at 22 hours after media change. In the experimental plate that received passive stretch in the Cytostretcher, media sample was collected 12 hours after completion of stretching protocol. This experimental design replicated three times and the level of muscle-injury biomarkers in eight biological samples (N=8) were quantified to evaluate the effects of passive stretch-induced injury before and after stretch.

3.5.1 Principle of the Assay

MSD^{R} provides a rapid and convenient method for measuring the levels of protein targets within a single, small volume sample. The assays in the Muscle Injury Panel 1 rat kit (K15181C-1) are sandwitch immunoassays. Sandwitch immunoassay requires two antibodies specific for different epitopes of the antigen. These two antibodies are 1) capture antibody that facilitates immobilization of antigen and is coated on the surface of plate, 2) detection antibody conjugated with the electrochemiluminescent tag which binds to analytes. The detection antibodies in Muscle Injury Panel 1 rat kit (K15181C-1) are listed below:

1) SULFO-TAG Anti-rat cTnI Antibody

2) SULFO-TAG Anti-rat cTnT Antibody

3) SULFO-TAG Anti-rat FABP3 Antibody

4) SULFO-TAG Anti-rat MyL3 Antibody

5) SULFO-TAG Anti-rat sTnI Antibody

MSD^{R} provides a plate pre-coated with capture antibodies. The user adds the sample and a solution containing detection antibodies conjugated with the electrochemiluminescent MSD^{R} SULFO-TAG^{TM} labels over the course two-hours long incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrodes surface; recruitment of detection antibodies by the bound analytes completes the sandwich. The user adds an MSD^{R} buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD instrument where a voltage applied to the plate causes the captured labels to emit light. The instrument measures the intensity of emitted light to provide a quantitative measure of analytes in the sample (Figure 3.16).

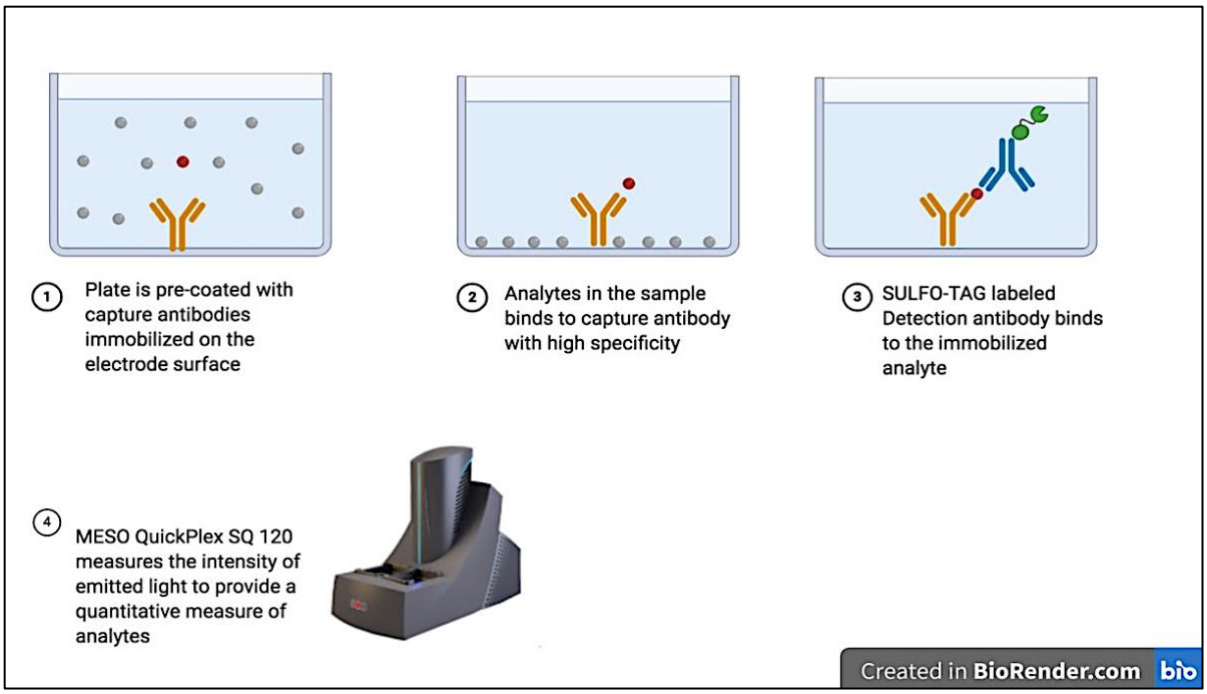


Fig.3.16. Principle of MSD^{R} electrochemiluminescence assay

2.5.2 MSD^{R} Muscle Injury Panel 1 Assays

Troponin is a protein the regulates muscle contraction in skeletal and cardiac muscle. Troponin acts with intracellular calcium to control the interaction of actin and myosin filaments in striated muscle fibers. When muscle tissue is damaged, the troponin-tropomyosin complex breaks down and troponin I and troponin T are released into the blood. MSD^{R} Muscle Injury Panel 1 kit provides three troponin assays (cardiac TnI, cardiac TnT, and skeletal TnI).

The three subunits of troponin are:

1) Troponin T is the subunit that interacts with tropomyosin to form the troponin-tropomyosin complex.
2) Troponin I is an inhibitory subunit that prevents muscle contraction in the absence of calcium. It is responsible for the binding of the troponin-tropomyosin complex to actin. Troponin I exists in three isoforms: slow twitch (striated) skeletal muscle, fast-twitch (striated) skeletal muscle, and cardiac muscle.

3) Troponin C binds calcium, producing a conformational change in troponin I and activating the troponin-tropomyosin complex.

Myosin light chain 3 (Myl3) is an essential light chain of the myosin molecule found in cardiac and slow-twitch skeletal muscle. The myosin molecule consists of a head domain that walks along the actin chain to contract the muscle and a tail domain that is responsible for binding myosin to its cargo. Two heavy chain subunits intertwine to form the head and tail domains. Four light chain subunits-two regulatory light chains with phosphorylation sites and two essential light chains bind the heavy chains together in the neck region between the head and tail domains. After damage to muscle tissue, myosin breaks down and Myl3 becomes elevated in the blood (Burch et al., 2015). Fatty Acid binding protein 3 (FABP3) is a cytoplasmic lipid transport protein that modulates uptake, transport and metabolism of fatty acids. In rat, skeletal muscle composed of primarily slow-twitch fibers have the highest concentration of FABP3, whereas FABP3 has lowest concentration in muscles containing fast twitch fibers (Pritt et al., 2008).

Chapter 3. RESULT

The purpose of this study was to characterize acute response of dystrophin-deficient and normal rat myofibers to passive stretch-induced injury *in vitro.* A commercial enzyme-linked immunosorbent assay kit (Meso Scale Discovery, Gaithersburg, MD, USA) was used to measure concentrations of skeletal muscle-specific injury biomarkers such as FABP3, Myl3, and STnI, at two time points, day three post differentiation, and 12-hours following completion of passive stretch protocol on day four post differentiation. The same measurement was repeated for control group (N=8) that did not receive passive stretch. Concentration of each injury biomarker was measured, processed, and further analyzed using DISCOVERY WORKBENCH^{R} software. Image J software was used to normalize the measured values to percentage of representative chambers' surface area covered by myofibers on day four of differentiation. Figures 3.17-3.19 illustrated Mean ± SEM concentration of FABP3, Myl3, and STnI measured on day three and four post differentiation in the four groups (DMD-Stretched, DMD-Control, WT-Stretched, and WT-Control). Two-way analysis of variance (ANOVA) with repeated measures test was selected to compare calculated mean between two time points within each group using GraphPad Prism 9. The level of statistical significance was set at $p<0.05$.

One hypothesis of this study was following the protocol of repeated passive stretches, the magnitude of injury was greater in dystrophic myofibers than normal. Based on Figure 3.17, STnI concentration was significantly higher on day four post differentiation compared to day three in the DMD-Stretched group ($P=0.0188$), whereas STnI concentration in all three groups did not show significant changes between the two time points. FABP3 concentration showed significant increase on day four of differentiation compared to pre-stretch (day three) in both DMD-stretched

(P= 0.0001) and WT-stretched (P= 0.0072) groups (Figure 3.18). However, MyL3 concentration decreased across all groups on day four of differentiation compared to day three (Figure 3.19). Overall, two injury biomarkers, FABP3 and STnI, demonstrated significant change between two time points in dystrophic myofibers, thus supporting the hypothesis that the passive-stretch protocol caused greater magnitude of injury in dystrophic myofibers compared to normal.

We further hypothesized that the absence of dystrophin as structural cytoskeletal protein exacerbates skeletal muscle injury and tested whether transduction of AAV6 micdystrophin into *mdx* myotubes could reduce the magnitude of stretch-induced injury. To test this hypothesis, AAV6 microdystrophin transduction was conducted similarly in both the DMD-control and the DMD-stretched groups on day of differentiation induction. Figures 3.20-3.22 presented Mean±SEM concentrations of FABP3, Myl3, and STnI biomarkers measured on days three and four post differentiation in the four groups (DMD-stretched, DMD-Control, WT-Stretched, and WT-Control). STnI concentration was significantly increased between two time points in the DMD-stretched group (P=0.0188), while the DMD-control group did not show significant change of concentration (Figure 3.20). However, FABP3 concentrations were not significantly different in both DMD-control and DMD-stretched groups (Figure 3.21). Similar to what noticed in the previous experiments, Myl3 biomarker showed pattern of decrease of concentration over time across all groups (Figure 3.22). The overall findings suggested that the passive-stretch protocol differentially affected STnI and FABP injury biomarkers responses in the myofibers treated with AAV6 microdystrophin transduction, thus it did not support the hypothesis that dystrophin-deficiency might worsen the response to passive-stretch induced injury.

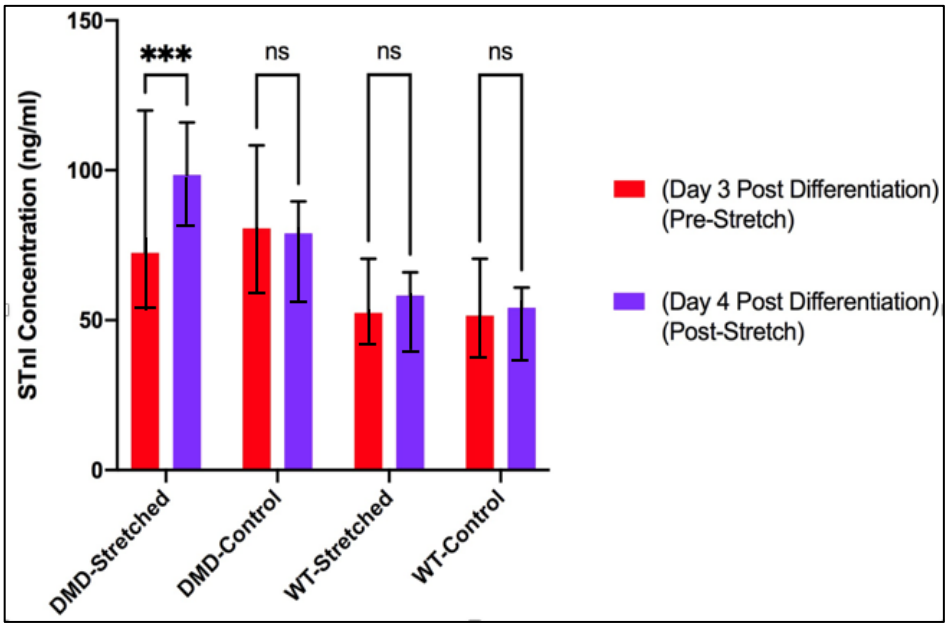


Fig.3.17. STnI concentration (ng/ml) (Mean ± SEM; N=8)
ns= Not significant (*P-value* > 0.05)
***= Adjusted *P-value* < 0.0001

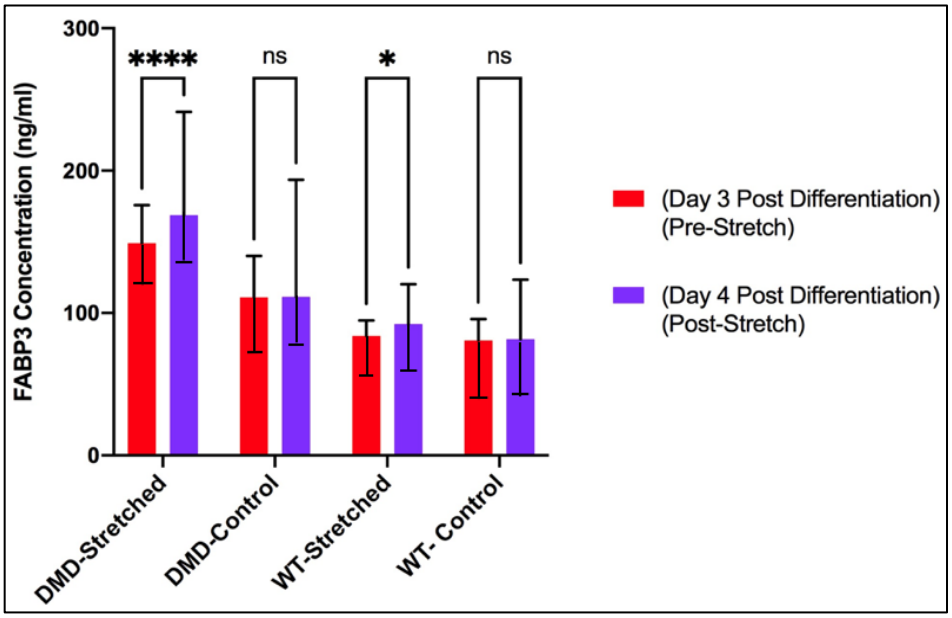


Fig. 3.19. FABP3 concentration (ng/ml) (Mean ± SEM; N=8)
ns= Not significant (*P-value* > 0.05)
*= Adjusted *P-value* < 0.05
***= Adjusted *P-value* < 0.000

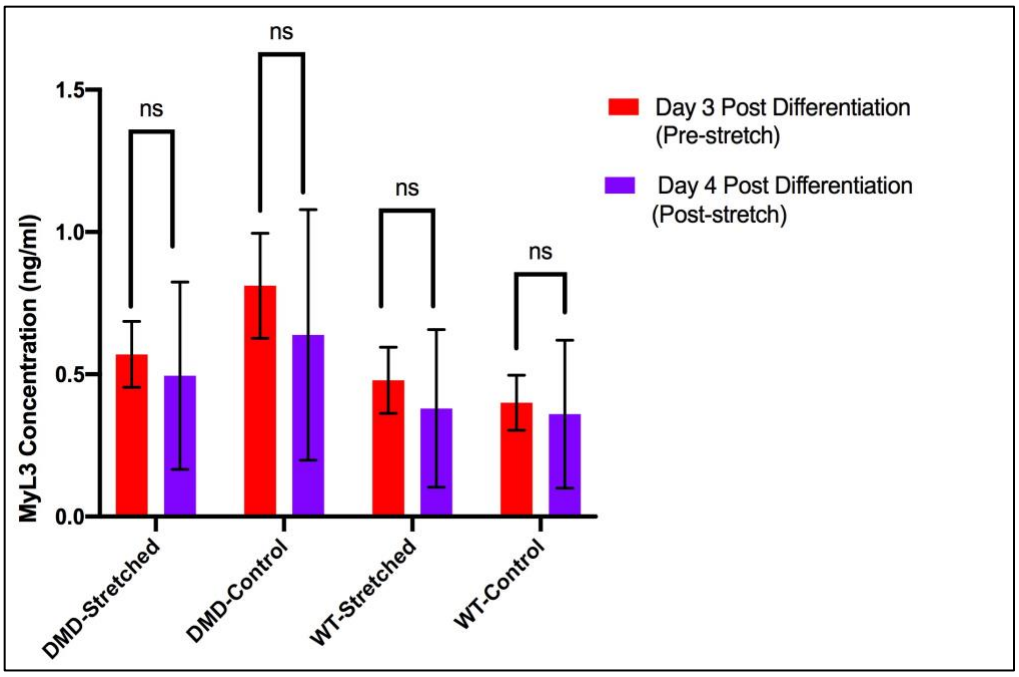


Fig. 3.20. MyL3 concentration (ng/ml) (Mean± SEM; N=8)
ns= Not significant ($P > 0.05$)

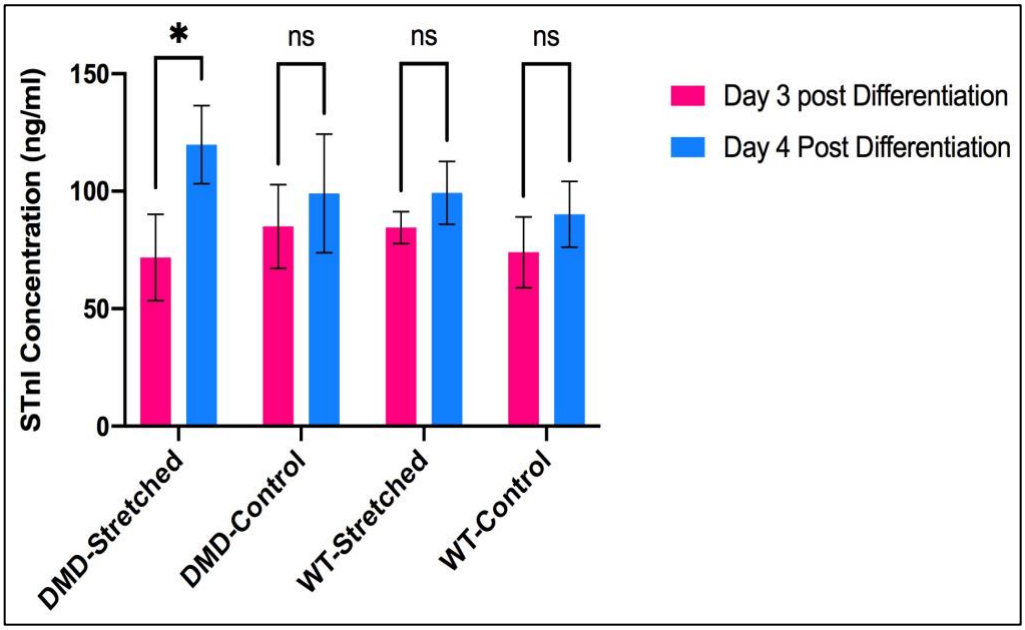


Fig. 3.21. STnI concentration (ng/ml) (Mean ± SEM; N=8); the DMD-stretched and the DMD-control groups were treated with AAV6 microdystrophin
ns= Not significant (*P-value* > 0.05)
*= *P-value* < 0.05

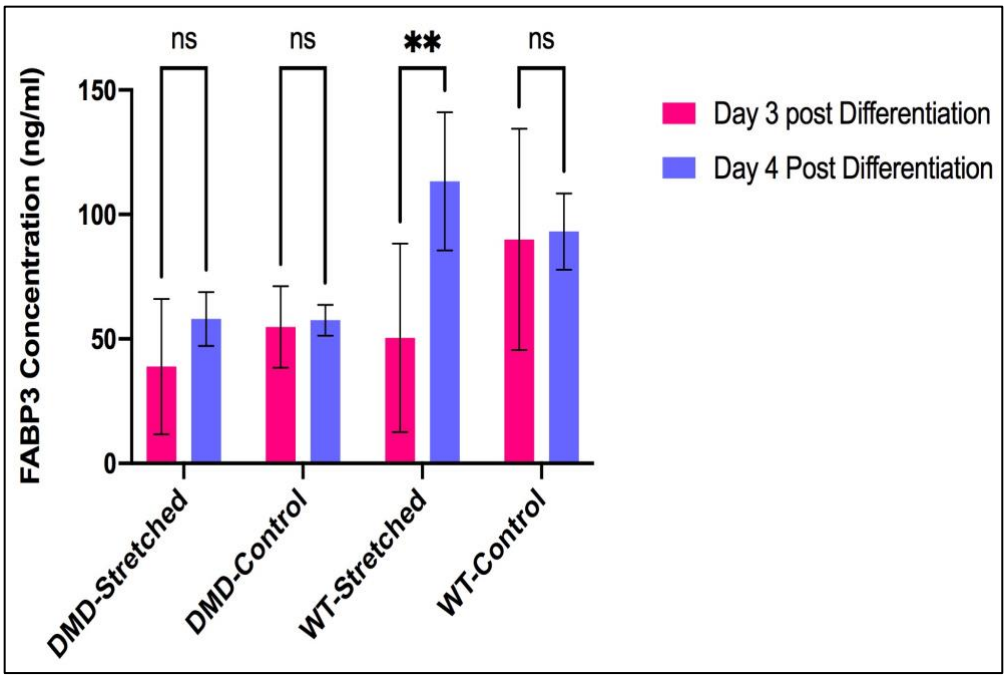


Fig. 3.22. FABP3 concentration (ng/ml) (Mean± SEM; N=8); the DMD-stretched and the DMD-control groups were treated with AAV6 microdystrophin
ns= Not significant (*P-value* > 0.05)
** =Adjusted *P*= 0.0049

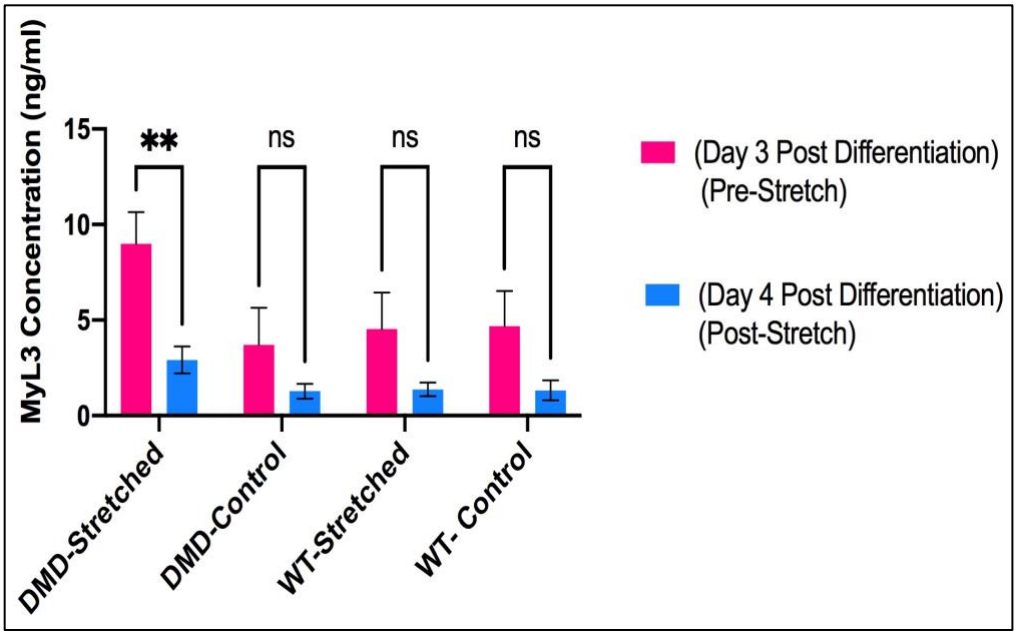


Fig. 3.23. MyL3 concentration (ng/ml) (Mean± SEM; N=8); the DMD-stretched and the DMD-control groups were treated with AAV6 microdystrophin
ns= Not significant (*P-value* > 0.05)
** =Adjusted *P* = 0.0072

Chapter 3. DISCUSSION

The main purpose of this study was to investigate the initial mechanisms responsible for passive-stretch induced injury in normal and dystrophic muscles. The study examined the effects of *in vitro* intermittent cyclic passive stretches of normal and dystrophin-deficient rat skeletal myofibers on acute biochemical response in order to reveal potential detrimental loading-specific characteristics of the protocol used. Most studies of mechanisms of stretch-induced injury are based on observations made either shortly after many repeated eccentric-contractions at the peak of fatigue, or days after, at the peak of delayed onset injury. As a result, conclusions based on these studies are complicated by interactions of mechanical and biochemical events, as well as the passage of time. Therefore, in a very fine-tuned AMS platform, confounding effects of muscle fatigue were minimized chemically to better study the initial biochemical events associated with passive stretch-induced injury. Although demonstration of morphological damage to myofibers is vital to confirm directly that an injury has occurred, the widely scattered nature of the damage makes a quantitative assessment of damage to fiber morphology difficult. Because of the limitations involved in a quantitative evaluation of the morphological damage and functional force measurement, we chose to focus on the direct association of the initial injury response with specific biochemical events within myofibers.

Using MSD^{R} enzyme-linked immunosorbent assay, concentrations of three skeletal muscle-specific injury biomarkers were measured and compared between two time points to determine acute biochemical responses of myofibers to passive stretch. Two injury biomarkers, FABP3 and STnI, demonstrated significant increase at 12-hour following the completion of passive stretch in dystrophic myofibers whereas only one biomarker, FABP3, showed significant

increase in normal myofibers. This preliminary finding suggested that the passive stretch protocol used in this study caused greater level of injury in dystrophic myofibers compared to normal, as evidenced by higher concentration of both cytoplasmic and myofibrillar regulatory proteins. This finding is in line with previous evidence that indicated muscle damage is greater and more easily induced in *mdx* compared to wild-type muscle fibers (Hamer et al. 2002; Whitehead et al. 2006).

STnI is known to be a specific and sensitive biomarker of skeletal muscle injury following exercise-induced muscle injury. It has been shown to have a broad diagnostic window with early onset of detection of 2-6 hours from onset of exercise-induced muscle injury, peaking at 24 hour and stays elevated for 1-2 days (Chapman, et al., 2013). The rapid breakdown of the troponin complex after stretch-induced muscle injury may be indicative of both membrane damage and degradation of the contractile proteins, thus suggestive of severe damage to muscle fibers. Moreover, in this study, MSD STnI assay is used as equally sensitive biomarker of injury in both fast and slow twitch muscle fibers and its concentration were shown to be higher at two time points in both the DMD-stretch and the DMD-control groups compared to the WT-stretched and WT-control groups. Thus, this finding is reinforced by previous studies that proved fast twitch muscle fibers are preferentially affected in early DMD pathology (Webster et al., 1988) and have higher susceptibility to injury than slow oxidative muscle fibers.

Previous studies described that fast-glycolytic muscle fibers are more susceptible to injury than slow oxidative ones. Possible reasons are explained for the differential susceptibility of fast muscle fibers to contraction-induced injury. First, the susceptibility relates to the metabolic differences between these fiber types, low oxidative capacity of fast glycolytic fibers may predispose them to injury during repetitive contractions through depletion of high-energy

phosphates and subsequent formation of high-energy actin-myosin cross-bridges. The breaking of these cross bridges through mechanical loading would then load to muscle injury (Macpherson et al., 1996). In addition to metabolic differences, structural differences between slow and fast fibers play role in their response to injury. For example, slow muscle fibers appear to contain higher levels of certain cytoskeletal proteins that may provide structural support for the sarcomeres and the cell membrane and may help to maintain integrity of these structures in the face of repeated mechanical loading. Moreover, the levels of other protective molecules, such as heat shock proteins are higher in slow than in fast muscle fibers which further provide protection from mechanical stress (Koh, 2002).

MSD MyL3 assay, a myofibrillar damage biomarker which is predominantly found in slow twitch fibers, did not show significant change in both the DMD-stretched and the WT-stretched groups, finding might suggest the differential sensitivity of myofibrillar proteins response to the stretching protocol used in this study. Future *in vitro* studies are needed to better understand the myofibrillar proteins response specially Myl3 to stretch-induced injury. On the other hand, cytoplasmic protein like FABP3 could be more directly released through a rupture in the sarcolemma compared to myofibrillar proteins (Mroczek, et al, 2019). Our finding revealed significant increase of FABP3 concentrations in the DMD-stretched group compared to the DMD-control group. Also, in the WT-stretched group FABP3 concentration was significantly increased after stretch compared to pre-stretch. This preliminary data suggested the potential detrimental loading-specific characteristics of the injury protocol used in both DMD and WT myofibers. However, cytoplasmic proteins do not necessarily reflect the amount of muscle fiber structural damage and are limited by their weak correlations with the degree of muscle fiber damage.

Therefore, myofibrillar proteins are needed to be studies along with cytoplasmic proteins to better understand the role of biochemical factors in skeletal muscle injury.

In this preliminary study, transduction of AAV6 microdystrophin into dystrophin-deficient myofibers did not reach convincing evidence as for reduction of magnitude of stretch-induced injury. Two possible explanations for the inconclusive findings are; 1) the insufficient dose of AAV6 microdystrophin (MOI= 10,000); 2) short duration of transduction. Therefore, optimization of the culture conditions such as using higher (MOI=20,000) for efficient lentiviral transduction and subsequent staining of dystrophin to confirm the quality of transduction is recommended for future studies.

In dystrophic muscles with weakened membrane integrity due to a lack of dystrophin expression, it is assumed that muscle-derived proteins are released in greater amounts than healthy muscle into circulation from membrane tears caused by mechanical injury during muscle contraction. However, this "leaky" muscle membrane hypothesis, have been challenged by researchers (Allen & Whitehead, 2011; Forcina et l., 2017). Moreover, findings of studies comparing the repair of *mdx* and control wild-type muscle fibers showed no difference in their ability to repair sarcolemma damage (Bansal et al., 2003; Duguez et al., 2013). Taken together, the rate and mechanism by which muscle-derived proteins are released may be both biomarker and disease dependent.

Traditionally, skeletal muscle damage has been assessed by the classical biomarkers creatine kinase (CK), aspartame amino transferase, and to a lesser extent lactate dehydrogenase (LDH) (Goldstein, 2017; Rodrigues et al., 2010). However, these biomarkers are nonspecific to skeletal muscle (Castro and Gourley, 2012; Keltz et al., 2014), lack sensitivity to detect lower

grade myopathies and do not correlate with disease severity in genetic myopathies (Govoni et al., 2013). This limits their ability to predict drug myotoxicity and to indirectly report drug effects on muscle function and recovery from injury. The MSD novel skeletal muscle injury panel of biomarkers consisting of STnI, FABP3, Myl3 has been identified as more specific and predictive of skeletal muscle injury. Previously, this biomarker panel has been widely characterized and validated in rodent models against multiple known myotoxicants including statins and clofibrate (Bodie et al., 2016; Burch et al., 2016; Maliver et al., 2017). Moreover, the MIP has been recently validated in patients with genetic muscular diseases (Burch et al., 2015; Goldstein, 2017; Hathout et al., 2016). Therefore, the ability to develop *in vitro* AMS model systems that can serve as a reliable platform to probe drug-induced myotoxicity and performance assessment of novel injury biomarkers to guide preclinical and clinical drug development studies is highly desired.

Given the complexity of the *in vivo* myogenic adaptation in response to exercise-induced loading, *in vitro* AMS models are crucial for understanding the cellular and molecular mechanisms that mediate loading-induced adaptations. The advantage of the *in vitro* model over others is that the forces imposed upon muscles or fibers during the injury protocol can be more accurately determined. Furthermore, in the *in vitro* models, muscle or fiber length and lengthening velocity can be more precisely controlled, and there are no or minimal anesthetic effects on contractility. The *in vitro* models are best suited for probing the cellular and subcellular mechanisms of injury in which it is possible to measure intracellular and extracellular ion, metabolic, and protein concentrations. Moreover, one has precise control over the muscle's environment and can add drugs or compounds to the solution. The main disadvantage of the *in vitro* models is the non-physiologic nature of the muscle's or fiber's environment. Because there is no vascular perfusion, only relatively small rodent muscles can be studied, and then for only a relatively short time. This

means that one can use the *in vitro* preparation to study only the initial injury events, not those occurring during the inflammatory and regenerative phases.

Future optimization of this *in vitro* injury model is highly recommended to enhance the physiological nature of the model in order to better address unmet clinical needs such as maximizing the therapeutic potential of eccentric exercise training and facilitation of identification of novel exercise-responsive drug targets. Furthermore, this *in vitro* injury model has the potential to guide development of future studies for drug discovery, such as blockers of stretch-activated channels to prevent increases in intracellular Ca^{2+} hold potential as therapeutic agents to overcome irreversible muscle damage in patients with DMD. Lastly, future research should be directed towards applying this *in vitro* model to study the chronic molecular mechanism controlling muscle adaptation, repair and regeneration. Studying the mechanisms responsible for chronic adaptation to acute exercise-induced muscle damage, particularly to eccentric exercise and/or passive stretch, is imperative for understanding how eccentric training can directly or indirectly alters normal muscle function in patients with DMD.

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Regenerative Management of Knee Chondral and Meniscal Lesions with Autologous Platelet Rich Plasma (PRP) and Bone Marrow Aspirate Stem Cells (BMACs) Combined with Rehabilitation

Abstract

Introduction: Orthobiologics are regenerative therapies that are new to rehabilitation settings. To achieve optimal outcomes clinicians must develop an understanding of their therapeutic role in the rehabilitation continuum. Orthobiologics, a rapidly growing treatment in orthopedic rehabilitation settings, introduce a paradigm shift in patient-centered care promoting regeneration and joint restoration by using blood-based procedures such as Autologous Platelet Rich Plasma (PRP) and Bone Marrow Aspirate Stem Cells (BMACs). The purpose of this pilot study was to examine the feasibility and short-term efficacy of the orthobiologics rehabilitation program following one PRP and/or BMACs injection on knee joint functional performance.

Method: Subjects with history of degenerative knee meniscal lesion or osteoarthritis, who had a history of failed physical therapy and underwent PRP and/or BMACs injections were referred by the physiatrist specialized in Orthopedic Regenerative Medicine to the Center for Rehabilitation and Clinical Research. Diagnosis of chondral and meniscal degeneration was confirmed by diagnostic Ultrasound and/ or MRI. Participants were selected based upon the inclusion criteria of having at least three orthobiological rehabilitation sessions post injection. Individuals with any concurrent hip joint osteoarthritis and/or active lumbar radiculopathy and previous histories of regenerative injections were excluded. A total of 12 patients who received PRP (N=5) or BMACs combined with PRP (N=7) in unilateral knee joint, were selected. The orthobiologics rehabilitation

protocol started with pain and swelling management, appropriate rest, no use of anti-inflammatory medications or modalities such as ice and electrical stimulation, manual therapy, correcting biomechanical maladaptive changes, and minimal graded mechanical loading in the initial inflammatory phase. Then, the protocol proceeded to moderate mechanical loading with closed chain and rotational stabilization exercises in the proliferation phase and progressed to dynamic closed chain exercise and knee joint stabilization exercises.

Results: The patient-reported outcome measure of Knee Injury and Osteoarthritis Outcome Score (KOOS) was used to analyze five different domains including Pain, Symptoms, Activities of Daily Living (ADL), Sport and Recreation Function (Sport/Rec) and knee-related Quality of Life (QOL). KOOS scores were collected at three time points; 1, 2, and 3 months post injection. The results showed the orthobiologics rehabilitation program was clinically meaningful across most KOOS subscales between 2 and 3-months post injection in both groups. However, participants in the BMACs combined with PRP injection group showed overall greater improvement of knee performance compared to one that only received PRP.

Conclusion: The preliminary findings of this small pilot study showed that the rehabilitation program was feasible following orthobiologics injection and had the potential to facilitate the healing process of knee in patients with history of knee chondral and meniscal lesions. Future clinical rehabilitation trials are needed to fully understand the role of rehabilitation following orthobiologics injection.

Chapter 4. INTRODUCTION

The American Physical Therapy Association defines Regenerative Rehabilitation as "the integration of principles and approaches from rehabilitation and regenerative medicine with the ultimate goal of developing innovative and effective methods that promote the restoration of function through tissue regeneration and repair" (American Physical Therapy Association [APTA], 2017). As regenerative therapy aims to facilitate connective tissue healing to prevent future injuries and maximize functional performance, it's use is becoming more common in orthopedic and sports medicine, particularly in the field of Physical Therapy. Regenerative rehabilitation pairs exercise principles (e.g., loading, intensity, frequency, duration), with regenerative therapies to facilitate regeneration and repair of musculoskeletal tissues (Glatt et al., 2019). This pilot study briefly introduced two commonly used orthobiologics injection used for treatment of degenerative knee disorders and evaluated the short-term effects of the orthobiologics rehabilitation program on knee functional performance at 1, 2, and 3 months post injection.

Phases of Tissue Healing

To understand tissue regeneration, it is important to consider tissue healing process after an injury, which includes three stages: the inflammatory, proliferative (repair) and maturation (remodeling) phases. In the inflammatory stage, the injured site fills with blood cells such as granulocytes which help remove injured cells and necrotic debris. During the proliferative phase, the native tissue cells replicate to replace the injured or damaged tissues. However, the initial tissue proliferation phase is disorganized and physiologically unstable which can lead to scar tissue formation. In the remodeling phase, the disorganized tissue cells form more organized structures and recover their physiologic properties. The healing phase may be impaired if the injured tissue remains in the inflammatory and proliferative stages and cannot enter the maturation phase to

restore join intrinsic physiologic properties, as seen in osteoarthritis (OA). The disrupted healing cycle promotes persistent cartilage breakdown by chronically releasing free radicals and activating proinflammatory mediators, such as matrix metalloproteinase and cytokines (Rand, E., & Gellhorn, A. C., 2016).

Articular Cartilage Regeneration

Articular cartilage damage can be caused by sport injuries, trauma, or aging. It can lead to more serious pathology like OA and subchondral bony edema. Articular cartilage has extremely poor capacity to repair and regenerate as cartilage is largely avascular, impairing repair responses. Chondrocytes have a limited potential for replication, which contributes to the limited intrinsic healing capacity of cartilage in response to injury, and their survival and function. Moreover, they exist in an environment that does not support healing, because they are surrounded by lacunae and cannot migrate to damaged areas and initiate repair processes (Johnstone, B., et al, 2013). Therefore, even minor insult to a joint can lead to progressive damage or joint degeneration.

Moreover, degeneration of the cartilage is due mainly to changes in activity of the chondrocytes to catabolic activity which lead to edema of the subchondral bone and inflammation of the synovium. In previous studies, it is demonstrated that chondrocytes that are located on the superficial region of articular cartilage produces cytokines such as interleukin-1(IL-1) and tumor necrosis factor (TNF) along with six different matrix metalloproteinases which together contribute to the intrinsic process of cartilage degeneration by resident chondrocytes (Hulejova, et. al, 2007) (Tetlow, L. C., et.al, 2001). Matrix metalloproteases (MMPs) are responsible for significant catabolic activity in cartilage via cleaving of extracellular matrix proteins, such as aggrecan and collagens. Moreover, the synthesis of new ECM in damaged cartilage is very slow and damaged cartilage is usually replaced by a cartilage that is histologically more resembles fibrocartilage than

hyaline cartilage and will be stiffer than original cartilage (Fosang & Beier, 2011; Mobasheri, et.al, 2014).

Knee Meniscal Regeneration

The healing potential of a meniscal tear is largely dictated by the location of the lesion. The precise mechanism by which a meniscal regeneration occurs remains unknown, it is, however, thought to occur via both extrinsic and intrinsic pathways (De Albornoz & Forriol, 2012). The extrinsic pathway is dependent on the tear site vascularity, where undifferentiated MSCs and endogenous growth factors interact and mediate repair. In fact, after meniscal injury the number of MSCs in the synovial fluid increases providing endogenous cells required for repair. The direct intrinsic pathway occurs via the self-healing capability of the remaining meniscal tissue and is not always a strong contributor to repair Mechanical loading is another factor which affects meniscal healing considerably and has undesirable effects on healing when the meniscus is loaded pathologically (Chew et al, 2017). Normal physiologic loading of the meniscus has been shown to have anti-inflammatory and overall anabolic effects while pathological loading, such as shear force has the exact opposite effect increasing catabolism, inflammation, and cell death (McNulty, & Guilak, 2015).

Chondrocyte and Mechanotransduction

Articular chondrocytes live in a dynamic environment with different forces that regulate genetic responses during physiologic joint loading. Type and intensity of mechanical loading directly affects how chondrocytes respond to mechanical loading. For example, dynamic, cyclic compression, and hydrostatic pressure can upregulate the transcription and translation of ECM proteins, such as aggrecan and glycosaminoglycan, whereas static compression downregulates it

(Roos, E. M., & Dahlberg, L, 2005). In addition to its direct effects on matrix proteins, mechanical loading can affect matrix homeostasis by interacting with inflammatory and catabolic signaling pathways. Moderate activity levels act as an anti-inflammatory signal by suppressing the inflammatory cytokine interleukin-1 (IL-1) and other inflammatory mediators by inhibiting MMP production, thereby limiting matrix breakdown (Ferretti, M., et al, 2006). Within a moderate range, MMP-1 showed significant, rapid, and sustained reduction as a result of exercise in various *in vitro* and animal experiments, and concomitant up-regulation of anti-inflammatory interleukin IL-10 (Helmark, I. C., 2010) (Madhavan, S., et al 2006). On the other hand, high levels of mechanical loading can result in the opposite effect, as evident in enhanced actions of IL-1 and MMP expression (Brisby, H., et al, 2010).

Orthobiologics

The American Academy of Orthopedic Surgeons defines Orthobiologics as biological substances found naturally in the body that help injuries to heal more quickly (Chu, et al, 2019). Intra-articular injection of orthobiologics has been introduced as a novel non-operative therapeutic method for knee degenerative disease and meniscal lesions (Huebner et al., 2018). The aim of cell-based therapies for cartilage defects is to repair damaged joint surfaces with a functional tissue capable of withstanding the stresses and strains of joint loading. Moreover, it can also be used to reverse degenerative processes, even if it cannot be fully regenerated, such as cartilage in OA (Jayaram et al., 2019). Understanding orthobiologics procedures and how tissues respond to mechanotransduction will help guide the development of appropriate rehabilitation programs for each type of regenerative therapy. In this study, two cell-based therapy approaches in orthopedic clinical setting and their applications in degenerative knee injuries are described. The pilot-study

tested feasibility and short-term efficacy of an orthobiologics rehabilitation program following PRP and/or BMACs injection on knee joint functional performance.

Platelet-rich Plasma (PRP)

PRP is defined as a plasma sample with platelet concentrations more than the baseline blood values processed from autologous blood. This platelet concentrate, rich in growth factor and nutrients, is injected to enhance natural healing response of an injured tissue. Platelets initiate healing by actively secreting seven fundamental protein growth factors: insulin-like growth factor (IGF), transforming growth factor (TGF-ß), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and connective tissue growth factor (CTGF) (Dhurat, & Sukesh, 2014).

Overall, PRP therapy aims to provide a favorable environment to recruit progenitor cells to orchestrate the interaction of cytokines and growth factors to stimulate the natural healing response for successful healing and return of normal strength, range of motion, and function. PRP plays a role in chondrocyte upregulation and cartilage matrix synthesis by the release of growth factors, in particular TGF-*ß*. TGF-*ß* is known to be a chondrocyte anabolism, initiating type II collagen synthesis and stimulating proliferation and chondrocyte differentiation into cartilage (Zhu, Y., et.al, 2013).

Very few studies have evaluated the use of PRP for meniscal lesions. *in vitro* or animal studies demonstrated that growth factors in PRP enhance the healing properties of the inner avascular part of rabbit meniscal cells and the biological activities of these cells for meniscal tissue regeneration (Ishida et.al, 2007; Zellner J. et al, 2014). A small retrospective study showed the potential of PRP injection to relieve pain, facilitate return to sport, and stop the progression of injury over the 6 months for grade 2 meniscal injuries (Blanke et. al, 2015). Comparative clinical

studies have demonstrated that autologous PRP injection has greater efficacy than hyaluronic acid and steroid injections in reducing pain and recovering articular function, especially for younger patients and milder knee OA (Spaková T, et. al, 2012; Vaquerizo, V., et. al, 2013; Raeissadat, S. A, et.al, 2015). In a randomized clinical trial, Patel et.al (2013) reported a significant improvement at 6 months in the WOMAC score in patients with knee OA who treated with one PRP injections while no difference between single or multiple PRP injection from 3 to 6 months. Forogh et. al (2016) compared patients with knee OA who were treated with one PRP or corticosteroid injection, they concluded that pain relief, activities of daily living, and quality of life were better in PRP group at 2 and 6 months. Table 4.1 summarizes findings of studies that compared PRP treatment with other interventions in patients with knee OA.

Table 4.1: Comparative Studies of PRP treatment with other interventions.

Study	Type of Study	Intervention	Results
Gormeli et al., (2015)	RCT	PRP, HA, Saline	Significant improvement in IKDC and VAS in PRP compared with Saline and HA
Montanez-Heredia et al., (2016)	RCT	PRP vs. HA	Improvements in pain scales with PRP at 3 months
Patel et al., (2013)	RCT	PRP or Saline Injection	Significant improvement at 6 months in WOMAC in the PRP
Forogh et al., (2015)	RCT	PRP vs. Corticosteroids	Pain relief, ADL, and OoL improvement in PRP at 2 and 6 months
Vaquerizo et al., (2013)	RCT	PRP vs. HA	Higher WOMAC response in PRP at 24 and 48 weeks
Spakova et al. (2012)	Prospective Cohort Study	PRP vs. HA	Improvement of WOMAC and VAS at 6 months in PRP
Raeissadat et al. (2015)	Randomized Non-placebo Controlled Trial	PRP vs. HA	Significant improvement in WOMAC pain score pain at 12 months in PRP
Sanchez et al., (2012)	RCT	PRP vs. HA	Significant higher response rate in WOMAC at 6 months in PRP
Blanke et al. (2015)	Retrospective Study	PRP	Relief of pain, facilitate return to sport, and halt progression of injury over 6 months

Mesenchymal Stem Cell (MSCs)

Bone marrow derived MSCs are commonly called “Bone Marrow Aspirate Concentrate” that contains bone marrow derived mesenchymal stem cells, hematopoetic stem cells, platelets (containing growth factors), and cytokines. BMACs are heterogeneous but can differentiate into lineages of mesenchymal tissue, like chondrocytes, leading to cartilage repair. BMACs supports hematopoiesis through production of stem cell factor (SCF), interleukin (IL)6, lymphocyte inhibitory factor (LIF), granulocyte macrophage-colony stimulating factor (GMCSF), granulocyte-colony stimulating factor (G-CSF), or macrophage-colony stimulating factor (M-CSF) (Sampson, S. 2015).

As described earlier, chronic inflammatory state causes chondrocyte apoptosis by the persistent activation of proteolytic enzymes that occur secondary to an increase in the activity of pro-inflammatory cytokines (IL-1a, IL-1, and tumor necrosis factor-a) and a decrease in the activity of the anti-inflammatory cytokines (IL-4, IL-10, and IL-1a). Therefore, given that stem cells have immunomodulatory, pro-angiogenic, and anti-fibrotic characteristics, therapies such as BMACs are often used in conjunction with PRP (Gupta, P. K., et al, 2012; Sampson, S. 2015). Regeneration of the damaged meniscus by PRP may be triggered by: (1) secreting platelet growth factors which augment natural regenerative pathways; (2) releasing growth factors which have a chemotactic and mitogenic effect on BMACs; (3) initiating angiogenesis; and/or (4) stimulating stem cells to proliferate as well as differentiate. (Pak, J., et. al, 2014). A systematic review of literature published between 1980 and 2015 reported overall good to excellent outcomes from BMACs injections for the treatment of knee osteoarthritis and chondral defects, with low complication rates (Chahla, J. et.al, 2016). In a recent multicenter randomized controlled clinical trial, the efficacy of intra-articular injection of BMACs combined with platelet rich plasma for the

treatment of knee OA is currently under investigation. No adverse effects in phase II were reported after BMACs administration or for 12 months follow-up (Lamo-Espinosa et al., 2020).

To date, there are no standard guidelines for rehabilitation after orthobiologics injection in human subjects. There is a need to better understand the synergetic effects of physical therapy with orthobiologics injections on knee functional performance and establish a standard guideline for post injection rehabilitation. Therefore, the purpose of this small pilot study was to examine the feasibility and short-term efficacy of the orthobiologics rehabilitation program following one PRP and/or BMACs injection on knee joint functional performance.

Chapter 4. METHODS

Participants

This study received ethics approval (Protocol# 639) from the Institutional Review Board at the PearIRB™. A total of 12 participants were included who received either PRP (N=5) or BMACs combined with PRP (N=7) (Table 4.2) and referred to the Center for orthobiologics rehabilitation. PRP and BMACs injections were administered by one physician specializing in Orthopedic Regenerative Medicine. All the research conducted through the Napa Medical Research Foundation, a 501(c)(3) nonprofit organization, is fully funded through generous donations received from individuals and family foundations

The PRP and BMACs preparation protocols for all participants were identical. Participants were selected based on the inclusion criteria of having a history of one orthobiologic injection and at least three orthobiologics rehabilitation sessions post injection. Patients with any concurrent hip OA and/or lumbar radiculopathy and previous history of regenerative therapy injections were excluded.

Table 4.2: Participants Demographic Description

Participants Groups	Number of participants	Type of Diagnosis	Age (Mean± SD)	Sex
PRP	5	Meniscal Tear (N=2) Grade II Knee OA (N=3)	45±7	F (N=5) M (N=1)
BMACs combined with PRP	7	Meniscal Tear (N=3) Grade II Knee OA (N= 4)	50±5	F (N=5) M (N=3)

Orthobiologics Rehabilitation Program

The orthobiologics rehabilitation program was performed by one Physical Therapist over a three-month period. KOOS self-reported knee outcome measure were collected at 1, 2, and 3 months post injection. The orthobiologics rehabilitation protocols started with pain and swelling management, appropriate rest, knee joint taping, gentle active range of motion, correction of biomechanical maladaptive changes, and open and closed chain minimal graded mechanical loading in the initial inflammatory phase. The inflammatory phase takes approximately 0-3 days post PRP and 1-week post BMACs injection. In the inflammatory phase, no anti-inflammatory medications or modalities such as ice or electrical stimulation were prescribed. During the proliferation phase, the protocol was continued with manual therapy and further proceeded to moderate mechanical loading with closed chain and rotational exercises (week 4-8). Duration of this phase was 4 days-8weeks for PRP and 1-12 weeks for BMACs injection. Afterwards, in the knee join remodeling phase, the protocol was progressed to progressive dynamic loading and lower kinetic chain stabilization exercises. Remodeling phase typically starts at about 2 months and 4 months for PRP and BMACs injection, respectively.

Outcome measure

The Knee Injury and Osteoarthritis and Outcome Score (KOOS) was used to evaluate participants' performance over three months. KOOS is a patient-reported outcome measure developed with the purpose of evaluating short-term and long-term symptoms and function in people with knee injury and OA. The KOOS includes 42 items in 5 separately scored subscales: Pain, Symptoms, Activities of Daily Living (ADL), Function in Sport and Recreation (Sport/Rec), and Knee- related Quality of Life (QOL). Each subscale is scored from 0 to 100, with 0 indicating extreme problems and 100 representing no problems. Subscales allow for evaluation of separate constructs at all levels according to the International Classification of Functioning. Psychometric Properties of KOOS in patients with articular cartilage lesions has fulfilled the basic requirements for reliability, validity, and responsiveness in cartilage or meniscal repair patients (Bekkers, J. E. J., et.al 2009; Roos E. M. et. al, 2003). The minimum clinically important difference (MCID) changes for Pain (6-6.1), Symptoms (5-8.5), ADL (7-8), Sport/Rec (5.8-12), and (QOL 7-7.2) is established for chondral lesions (Collins, Misra et al. 2011). For the purpose of this small pilot study, MCID of KOOS sub scores within each group was reported and compared between the first three months post injections. Future statistical analysis of data with higher sample size using Analysis of Variance (ANOVA) with repeated measure test will be warranted to compare change of scores over time.

Chapter 4. RESULTS

Feasibility was evaluated as the percentage of participants who were able to complete orthobiologics rehabilitation program without adverse events (e.g., loss of balance, fall, joint pain, muscle soreness, fatigue). All participants completed the protocol with 100% attendance and no adverse events. In participants who received BMACs combined with PRP, mean KOOS scores

showed gradual improvement across all subscales over three months evaluation after injection. However, pain subscale showed an opposite trend between one month and two months post injection (Figure 4.1). In pain subscale of KOOS, 6 participants showed clinically meaningful improvement in pain between two and three months, while none of them showed clinically meaningful improvement between one and two months. In symptom subscale of KOOS, 4 participants demonstrated clinically meaningful improvement between one and two months, compared to two participant that presented improvement between two and three months. Under ADL subscale of KOOS, 4 patients showed improvement between one, two, and three months post injection, versus only 2 participants had clinically meaningful improvement between one and two months. Under Sport subscale, 5 participants had clinically meaningful improvement between one and two months, while 3 participants had clinically meaningful improvement between two and three months. QoL was clinically improved in 4 participants between two and three months, while only 1 participant had clinically meaningful improvement between one and two months (Table 4.3).

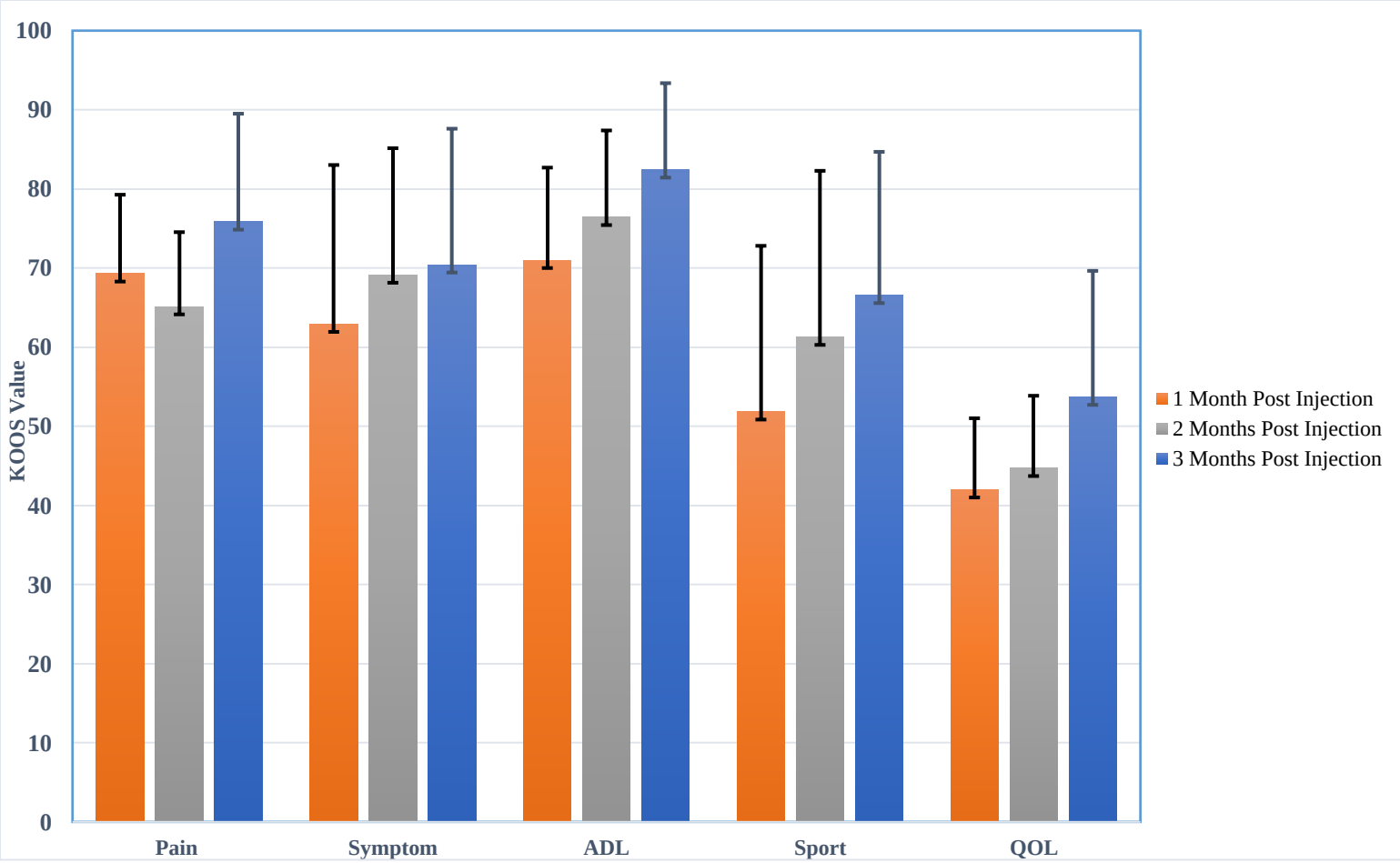

Fig.4.1: Mean and Standard Deviation of KOOS Subscales value in the BMACs Combined with PRP group (N=7)

Table 4.3: KOOS Subscales change of scores between one, two, and three months post BMACs combined with PRP injection

Participants	Pain (M-1-2)	Symptom (M-1-2)	ADL (M-1-2)	Sport (M-1-2)	QoL (M-1-2)	Pain (M-2-3)	Symptom (M-2-3)	ADL (M-2-3)	Sport (M-2-3)	QoL (M-2-3)
P-1	-7	-8	+1	+7*	0	+18*	+22*	+12*	+13*	+25*
P-2	-7	-6	-6	-15	-6	+4*	-8	+12*	+26*	0
P-3	+4	+22*	+2	+10*	0	+11*	-8	+12*	0	+12*
P-4	-7	0	+5	0	+13*	+12*	-1	+5	-5	0
P-5	-15	+ 9.5	+18*	+34*	+6	+19*	+15*	+9*	+23*	+13*
P-6	+2	+8*	+7*	+11*	0	0	+2	+2	-10	+13*
P-7	-5	+18*	+7*	+10*	+6	+11*	-13	-10	-10	0

M= Month; * = Change of scores between two time points was above established MCID values

In participants who received PRP injection, mean KOOS scores showed improvement across all subscales between two and three months after injection. However, all subscales except ADL showed exacerbation between one month and two months post injection (Figure 4.2). In pain

subscale of KOOS, 2 participants showed clinically meaningful improvement between one and two months, while 3 out of 5 showed clinically meaningful improvement between two and three months. In both symptom and Sport activity subscales of KOOS, 1 participant demonstrated clinically meaningful improvement between one and two months, compared to 3 participants who presented improvement between two and three months. Under ADL subscale of KOOS, 1 patient showed improvement between one month and two months post injection, versus only 2 participants had clinically meaningful improvement between two and three months. QoL was clinically improved in 3 participants between second and third months, while only 1 participant had clinically meaningful improvement between one and two months (Table 4.4).

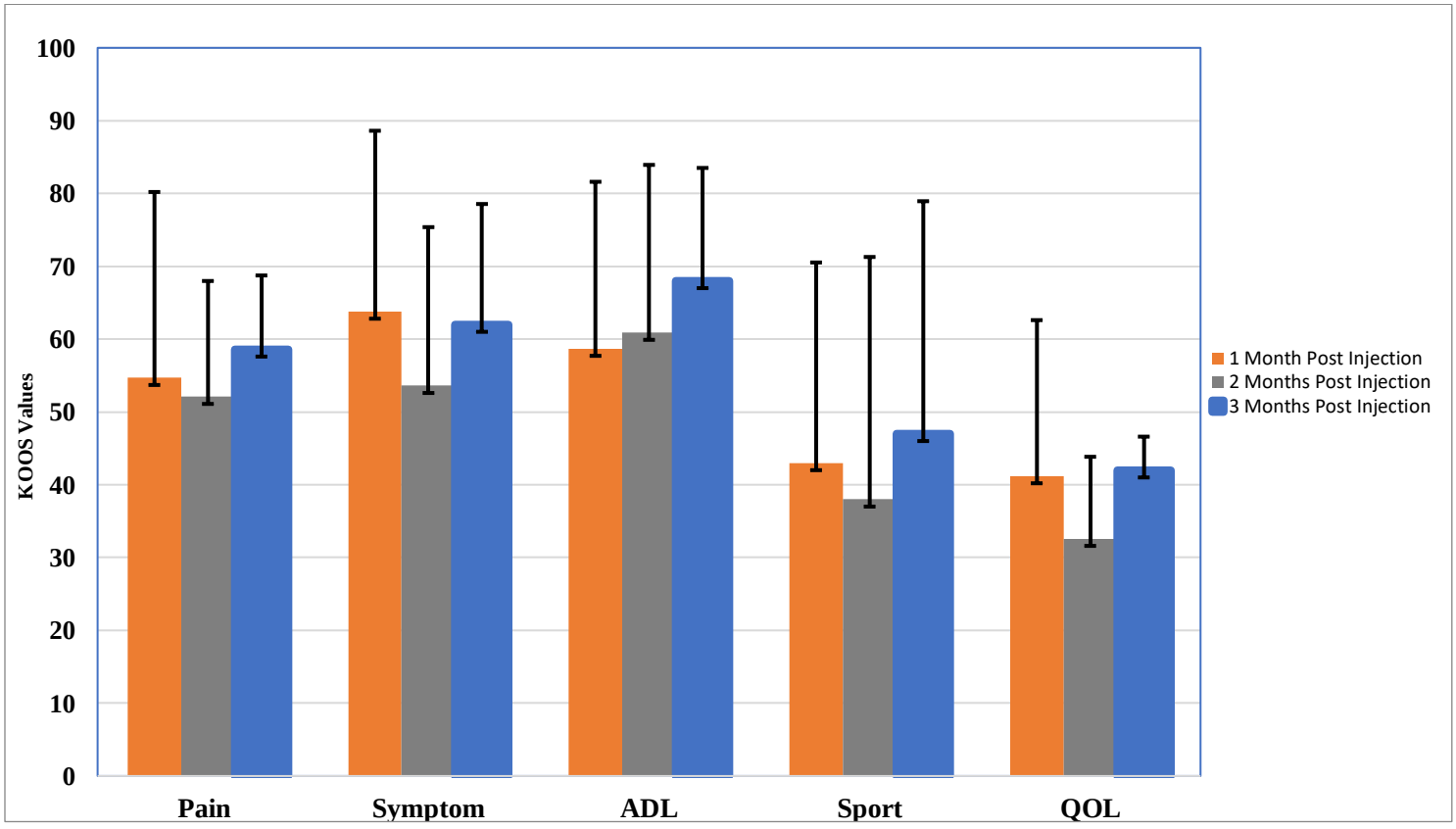

Fig.4.2: Mean and Standard Deviation of KOOS Subscales Value in the PRP group (N=5)

Table 4.4: KOOS Subscale change of scores between one, two, and three months post injection in the PRP Group

Participants	Pain (M-1-2)	Symptom (M 1-2)	ADL (M-1-2)	Sport (M- 1-2)	QoL (M- 1-2)	Pain (M- 2-3)	Symptom (M-2-3)	ADL (M- 2-3)	Sport (M- 2-3)	QoL (M- 2-3)
P-1	-30	-46	-4	0	+19*	-8	+2	+3	0	+6*
P-2	+4	-3	+11*	+30*	-6	+3	+6*	0	+10*	-2
P-3	+7*	-1	-1	-10	-6	+7*	-3	-6	0	0
P-4	-4	-6	0	-25	0	+8*	+23*	+19*	+10*	+13*
P-5	+10*	+5*	+5	-20	+6.5	+22.5*	+23*	+19.5*	+25*	+19*

M=Month; * Change of scores between two time points was above (MCID) values

Chapter 4. DISCUSSION

The purpose of this small pilot study was to examine the feasibility and short-term efficacy of the orthobiologics rehabilitation program on self-reported knee joint performance in patients with degenerative chondral or meniscal lesions. The preliminary results showed the orthobiologics rehabilitation program was clinically meaningful across most KOOS subscales between two and three-months post PRP injection (early remodeling phase). However, during the proliferation phase (1-8 week), exacerbation of symptoms might account for the inconsistent patterns of change observed among participants between one month and two months post injection. Previous clinical trials of knee OA treated with PRP injections without physical therapy, reported improvements of functional performance using WOMAC, which occured mainly at very late stage of remodeling phase of healing (6 months) (Patel et al., 2013; Blanke et al., 2015; Forogh et al., 2016) (Table 2). However, the initial findings of this study indicated that physical therapy program after PRP have the potential to accelerate functional outcomes as reported in KOOS.

Participants who were treated with BMACs combined with PRP injection in conjunction with physical therapy showed clinically meaningful improvement of knee scores at three months post injection. The observed improvement of KOOS scores were clinically meaning in more patients in the late stage of proliferation (2-3 month) compared to the early phase of proliferation (1-2 month)

(Table 2). Previous studies demonstrated improvement of pain scores and function at one-year post injection in patients who were treated with BMACs without physical therapy (Patel et al., 2013; Forogh et al., 2015). This preliminary finding suggested that synergistic effects of orthobiologics with rehabilitation has the potential to facilitate clinical outcomes in the remodeling phase.

A combined rehabilitative approach to improve biomechanics and promote stability along with the use of PRP and/or BMACs injection to induce healing may better assist in preventing progression of degenerative chondral lesions in knee joint. The key for patient functional recovery is in the combination of the two therapies, the autologous cellular injection with physical therapy. In combining the criteria of biologic healing time with recovery of lower kinetic chain biomechanics, physical therapy becomes an essential component of the regenerative healing process. Nuance of the combination of interventions provides promise for as an alternative treatment approach.

Future studies are required to comparatively investigate combined regenerative therapy injections and physical therapy vs. either treatment alone, or for multiple diagnoses (e.g. OA, meniscal injury, etc.) before recommendations for best practice can be made. Limitation of this study was small sample size, absence of control arm, and lack of objective measures. Combining objective clinical measures of knee strength and function with diagnostic MRI would allow researcher to better evaluate synergistic effect of rehabilitation post orthobiologics injection. Therefore, future randomized controlled studies are needed to investigate the short term and longitudinal efficacy of PRP alone or in combination with BMACS and physical therapy in order to inform clinical decision making.

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Mechanobiology is the foundation for any intervention that use mechanical loading of target tissue to employ its therapeutic benefits. Musculoskeletal system which is populated by mechanosensitive cells offers one of the best opportunities to study the effects of mechanotherapy. This system is highly responsive to changes in functional demands through modulation of mechantransductive pathways. In this hybrid document, mechanical loading of musculoskeletal system with focus on skeletal muscle cells and chondrocytes were briefly studied to better understand the role of mechanotherapy in biological tissue responses to loading.

First, mechanical loading of skeletal muscle cells in the form of passive stretch was used to model *in vitro* stretch-induced injury and then the response of normal and dystrophin-deficient myofibers to the model was further assessed. The preliminary findings suggested that dystrophin-deficient myofibers were more susceptible to the stretch-induced injury than normal based on their response to the selected passive-stretch protocol. Improvement of physiological aspects of this *in vitro* injury model is recommended for future design of studies to examine eccentric contraction-induced injury response in dystrophin deficient skeletal myofibers.

Second, mechanical loading of degenerated chondrocytes in knee meniscal and/or chondral lesions in the form of rehabilitation program was evaluated in the pilot study to understand the role of physical therapy intervention on the clinical outcomes of patients who underwent knee regenerative therapies. The field of regenerative rehabilitation has great potential to improve clinical outcomes for patients with disabilities. However, this field is currently in its infancy and needs rigorous scientific inquiry to begin to elucidate the biologic underpinnings of regenerative rehabilitation-based approaches. The role of mechanical loadings is well established in prevention of musculoskeletal complications following disease or injury, however optimal dosing of

mechanical loads and the timeline of when to use them during the repair process to produce the optimal effects is not fully understood. In fact, clinicians understanding of dosing is mainly based on the patient's response to intervention, yet there is a need to precisely load healing tissue with the exact frequency, duration, magnitude, and types of loads to optimize movement with minimal risk. Therefore, future research in the field of regenerative rehabilitation is necessary to include clinical implications of force specificity and load dosing in order to enhance current interventions.

APPENDICES

STATEMENTS OF CO-AUTHORSHIPS

Statement of Co-Authorship (1)

As co-authors of the manuscript entitled "**Intersection of Mechanobiology and Physical Therapy Practice in Skeletal Muscle Rehabilitation**" that had been prepared for submission to the Physical Therapy Journal,

We confirm that Maryam Fayazi was the primary contributor to the study in each of the following areas:

- Design of the study
- Data collection
- Data analysis and interpretation of the findings
- Writing of the manuscript and critical appraisal of the content

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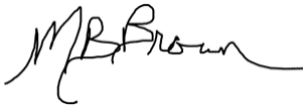
Mary Beth Brown		2-19-21
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Statement of Co-Authorship (2)

Statement of Co-authorship

Statement of Co-Authorship

As co-author of the manuscript entitled "Regenerative Management of Knee Chondral and Meniscal Lesions with Autologous Platelet Rich Plasma (PRP) and Bone Marrow Aspirate Stem Cells (BMACs) Combined with Rehabilitation" that had been prepared for submission to the Journal of Sport Physical Therapy,

We confirm that Maryam Fayazi was the primary contributor to the study in each of the following areas:

- Data analysis and interpretation of the findings
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